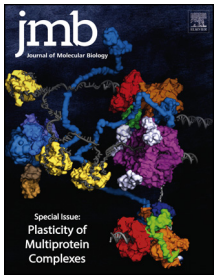
Review



Subunit Exchange in Protein Complexes

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Abstract

Over the past 50 years, protein complexes have been studied with techniques such as X-ray crystallography and electron microscopy, generating images which although detailed are static and homogeneous. More recently, limited application of *in vivo* fluorescence and other techniques has revealed that many complexes previously thought stable and compositionally uniform are dynamically variable, continually exchanging components with a freely circulating pool of "spares." Here, we consider the purpose and prevalence of protein exchange, first reviewing the ongoing story of exchange in the bacterial flagella motor, before surveying reports of exchange in complexes across all domains of life, together highlighting great diversity in timescales and functions. Finally, we put this in the context of high-throughput proteomic studies which hint that exchange might be the norm, rather than an exception.

Introduction

Life is change. Even in the human body, which seems outwardly stable, the microscopic picture is one of constant flux—ATP molecules turn over thousands of times per day, and a typical protein molecule lasts only a few days before being degraded [1, 2]. Many cell types have a lifetime of days, weeks or months—a fraction of the lifetime of the organism as a whole [1]. By contrast, the triumphant success of structural biology over the last 50 years in generating beautiful, compelling images of biomolecular complexes, offers a picture of static and highly stable structures. In particular, X-ray crystallography and electron microscopy (EM) have revolutionized our understanding of proteins and protein complexes, ranging in size from kiloDaltons to many tens of megaDaltons. The majority of these complexes are dynamic in structure, composition or both. X-ray crystallography, however, requires the selection of highly stable and homogenous complexes to generate atomic resolution, relegating structural dynamics and heterogeneity to a role somewhere between a minor inconvenience and a signature of failure. EM cannot match the resolution of X-ray crystallography, although recent improvements bring it ever closer [3]. In principle, cryo-EM and the related cryo-electron tomography (ET) [4] offer images of individual molecular complexes, static because frozen to cryogenic temperatures but preserving heterogeneity. In practice, however, images of single complexes have relatively low resolution, and there is a trade-off between preserving heterogeneity and increasing resolution by averaging many images. Atomic force microscopy (AFM) offers a tantalizing promise of combining resolution within single-protein complexes with observation of their working dynamics [5], but high-speed AFM remains technically extremely difficult and AFM is limited to samples that can be observed on hard flat surfaces.

Over the last decade, new single-molecule and *in vivo* biophysical methods have allowed for investigation of the stability of large protein complexes, working in their natural environment inside live cells. This has revealed that heterogeneity and dynamics in molecular composition are the norm. For example, in the bacterial flagellar motor (BFM), protein molecules in both the rotor and stator were observed to exchange with freely circulating pools of "spares" on a timescale of minutes, even in motors that were continuously rotating [6–8]. We will call this process

"protein exchange" and take care here to distinguish it from protein "turnover"—a term misleadingly used in some of the early literature on protein exchange. Exchange describes the binding and unbinding of intact protein components and complexes. Turnover, by contrast, is the destruction (digestion) and synthesis of proteins [9]. While exchange may be a prerequisite for turnover, the two processes are entirely different. This review addresses the question, "What is the role of protein exchange?" Is it most often merely a by-product of the relative weakness of protein–protein interactions? Or a necessary step in the turnover of damaged proteins? Or is it a mechanism for the regulation, modification and adaptation of complexes? Our definition of protein exchange requires us also to define what we mean by "protein complex." We limit ourselves in this review to consideration of complexes that are finite in size and sufficiently well defined and stable to have yielded an informed picture, however, incomplete, of their structures. This excludes, for example, transient complexes seen in cell signaling [10] and protein components that are assembled or disassembled deterministically in response to specific triggers. In Photosystem II, for example, an elaborate pathway removes and replaces the D1 subunit only in response to oxidative damage [11]. We also specifically exclude dynamic filaments and their accessory proteins, as are seen in the cytoskeleton—these have long been known to exchange and are extensively reviewed elsewhere. Such filamentous complexes are also free to grow indefinitely, unlike all the complexes we consider below which appear to be bounded.

We focus first on protein exchange in the BFM, a rotary electric motor ~ 50 nm in diameter composed of a few dozen types of protein. When we first uncovered the extent of subunit exchange in the BFM, we were astonished that such an intricate machine could operate continuously despite the regular exchange of essential components. This provoked questions about exchange more generally—how common is it, and what purposes does it serve? We even considered that it might be an unavoidable consequence of the flexibility and marginal stability of all proteins, or a mechanism for proofreading during assembly. In this article, we offer a perspective on how discoveries over the subsequent decade go some way toward answering those questions. We review the current understanding of exchange in the BFM, which exhibits some of the best-studied exchange processes of any complex. After discussing the injectisome, a close relative of the BFM, we then look at examples of protein exchange in other well-characterized complexes, highlighting the great diversity of complexes exhibiting exchange and the broad range of associated timescales and putative functions. We then put this in the context of results from high-throughput proteomics techniques, which give a less detailed but more impartial birds-eye view of exchange in the proteome in general. It appears that subunit exchange occurs in many complexes, in all domains of life, over a large continuum of timescales. Rather than being an essential feature, at least at shorter timescales, it may be an option exploited by nature for a wide range of different uses, many of which are only just beginning to be understood. Furthermore, while the extent of subunit exchange is still unclear, evidence of widespread dynamism in protein complexes generally points to it being widespread.

Protein exchange in the BFM and injectisome

BFM structure and function

The BFM is a membrane-embedded ion-driven rotary motor that powers bacterial swimming motility and surface motilities such as swarming [12, 13]. In *Escherichia coli (E. coli* and *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium), the best-studied model systems, multiple motors per cell each drive a 5- to 10-μm-long helical filament at speeds in excess of 100 Hz *via* a universal joint ("hook") that transmits torque to the filament while allowing freedom of the filament orientation [12]. The motor, a large complex (~ 11 MDa) incorporating at least 13 proteins in copy numbers ranging from a several to dozens, comprises a rotor surrounded by a ring of force-generating stator units—selective ion channels spanning the inner membrane and anchored to the cell wall.

Rotation is powered by ion motive force (IMF)—an electrochemical gradient consisting of the combined effect of concentration and voltage imbalances across the inner membrane which drive ion transit through the stator units into the cytoplasm (Fig. 1). This transit is coupled to conformational changes in the stator units which push against the rotor, generating torque [14, 15]. Stator units are stable sub-complexes of 4 MotA and 2 MotB proteins in H^+-powered motors of *E. coli* and *S.* Typhimurium, Fig. 2a) [16], or of their homologs such as 4 PomA and 2 PomB in Na^+-powered motors of *Vibrio alginolyticus*) [17]. H^+-powered and Na^+-powered stators [18, 19], along with more recently discovered stators powered by K^+/Rb^+ [20] and Ca^{2+}/Mg^{2+} [21], are very similar in sequence and function.

The flagellar rotor comprises a shaft coupled to a series of protein rings (Fig. 2a): the inner-membrane-spanning MS-ring (FliF) is the structural core, while the cytoplasmic C-ring [or switch complex, comprising FliG, FliM and FliN] forms the surface which is pushed by the stator units. The C-ring also hosts the cytoplasmic export apparatus, responsible for controlled export of substrates used to assemble the shaft (rod), hook and filament [25]. Averaged cryo-EM reconstructions of rotors purified as stable

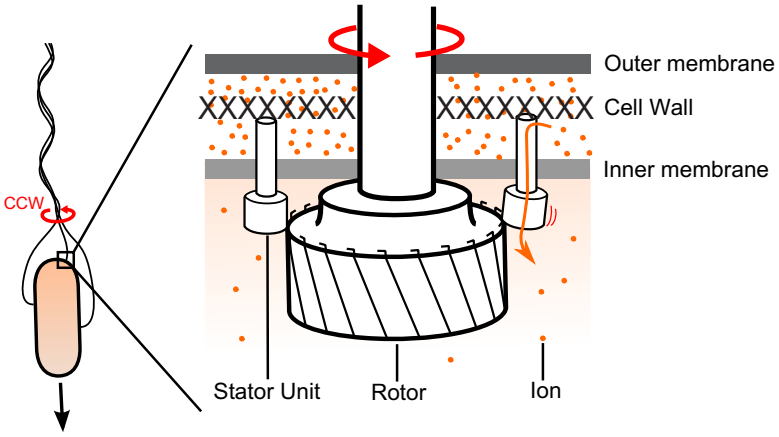

Fig. 1. Bacteria such as *E. coli* and *Salmonella* swim propelled by a bundle of rotating flagella (left). Each flagellum is powered by a rotary motor embedded in the cell envelope at its base (right). The flagellar motor is driven by the flux of ions, most often H^+ or Na^+, down an electrochemical gradient across the cytoplasmic membrane into the cell.

complexes, including C-ring but not export apparatus, have been central to our understanding of motor structure [26–28] (Fig. 2b, c). More recently, similar reconstructions at lower resolution by cryo-ET, of entire motors *in situ*, show that motors from different species share a structural core while varying in the stator and C-ring (Fig. 2d–f). Crystal structures of several component proteins and protein fragments have been solved.

In *E. coli* and *S.* Typhimurium, the C-ring can switch between two states, coupling the conformational change in the stator unit to either clockwise (CW) or counter-clockwise (CCW) rotation (defined looking from the filament toward the motor). When all motors in a cell are rotating CCW, all filaments form into a bundle which propels the cell smoothly (Fig. 3a). However, if a motor switches transiently to CW rotation, a conformational change is induced in the filament which expels it from the bundle, causing a "tumble"—a random change in swimming direction (Fig. 3b). The chemosensory apparatus of the cell controls the frequency of motor switching events to navigate chemical gradients in the cell's environment—chemotaxis (Fig. 3c,d). This is mediated by the diffusing cytoplasmic protein CheY, which in its phosphorylated state (CheY-P) binds to the C-ring and increases the probability of CW rotation (and therefore a tumble). When the cell is traveling in a desirable direction (e.g., the concentration of external chemoattractant is increasing), phosphorylation of CheY decreases, suppressing tumbling. Continuous adaptation of the chemosensory system updates the reference value against which increases or decreases of chemoattractant are judged on a timescale of a few seconds. Thus, the cell moves in a random walk biased toward chemoattractants and away from chemorepellants. While other species have different chemotaxis strategies, some of which rely on changes in motor speed rather than direction, CheY-mediated modulation of motor activity, presumably *via* C-ring binding, is common to all those which have been well-studied [29–31].

Early observations of protein exchange

The BFM is a canonical example of a molecular machine, and the default assumption in the early days after its discovery was that once assembled, each motor is essentially immortal and unchanging. However, measurements of the rotation of single motors in the 1980s provided the first indication of structural dynamics in the BFM [32–36]. When tethered to a surface *via* a single flagellar motor, rotation of the cell body allows for easy measurement of motor activity. In "resurrection" experiments, the expression of stator proteins from an inducible plasmid restored rotation in a series of up to 8 discrete speed increments in motors previously lacking functional stator proteins. This was interpreted as the sequential assembly of 8 stator units into the motor, each contributing independently and additively to rotation, and demonstrated that motors could rotate during assembly. Transient stepwise speed decreases were also seen, now attributable to protein exchange [32, 36]. More recently, up to 11–12 speed increments have been seen in *E. coli* BFMs (Fig. 4a) [34], again accompanied by occasional stepwise speed decreases.

In 2006, we performed the first direct observation of stator unit exchange [6], using fluorescence recovery after photobleaching (FRAP) and fluorescence loss in photobleaching (FLIP). These techniques had long been used to measure protein exchange in the cytoskeleton [37], and more recently in non-cytoskeletal protein complexes [38], but never to single complexes and never with simultaneous recording of the function of that complex—motor rotation, in our case. Cells with stator protein MotB fused to green fluorescent protein (GFP), introduced by chromosomal replacement to ensure natural expression—were imaged with total internal reflection fluorescence (TIRF), which selectively excites fluorophores within ~100 nm of the coverslip, encompassing the motor and cell membrane, but excluding most of the cytoplasm (Fig. 4b). Motors in tethered cells were

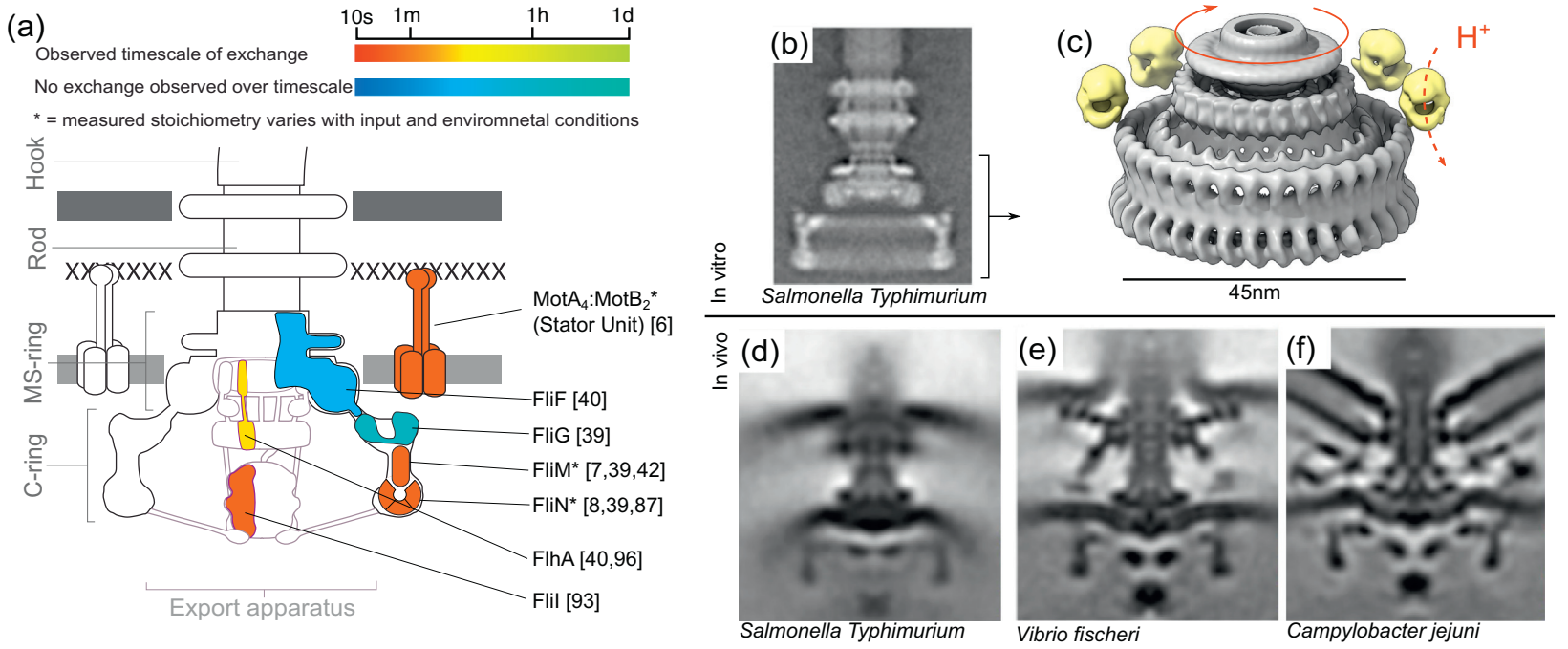

Fig. 2. (a) BFM structure and composition. Colors indicate published measurements of subunit exchange. Figure adapted from Ref. [12]. (b) EM average of rotors purified from *S.* Typhimurium [22]. (c) EM reconstruction of FliF and C-rings of rotors purified from a *S.* Typhimurium mutant with CW-locked motors (gray), surrounded by EM reconstruction of MotA tetramers (partial stator units) from *Aquifex aeolicus* (yellow) [23]. (d–-) Sub-tomogram averages of motors from *S.* Typhimurium, *Vibrio fischeri* and *Campylobacter jejuni*, imaged *in vivo* with cryo-ET [24].

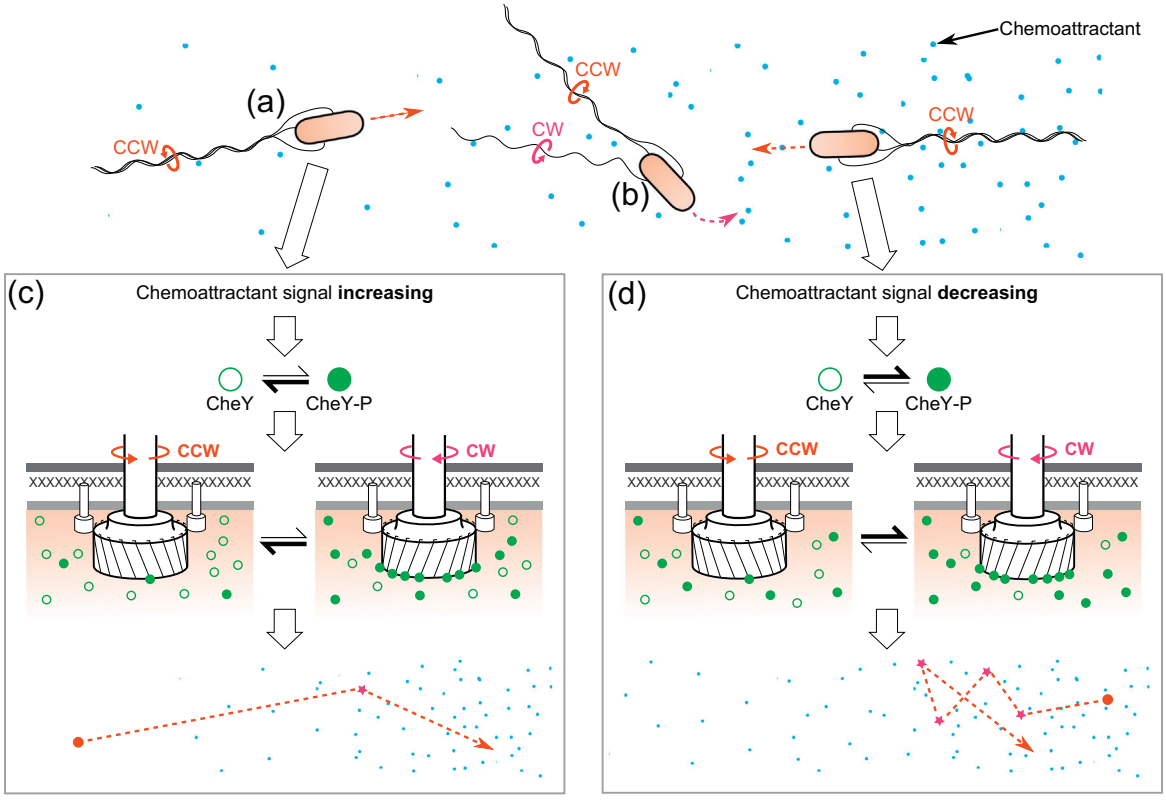

Fig. 3. Motor switching and chemotaxis. (a) When all motors rotate CCW, filaments form a bundle, propelling the cell smoothly. (b) If one or more motors rotate CW, filaments are ejected from the bundle, causing a "tumble"—a random change of direction. (c, d) Chemotaxis results from the regulated phosphorylation of CheY, which controls motor switching: increased binding of chemoattractant to a sensory cluster at the cell pole suppresses the phosphorylation of CheY and associated CW rotation and tumbling, prolonging runs in the direction of increasing attractant concentration (c), and *vice versa* (d).

visible as fluorescent spots at the center of cell rotation. Motors were also observed in non-rotating cells, with more than one spot visible in TIRF indicating the likelihood of two or more tethered motors blocking rotation.

For FRAP experiments, an intense laser spot was focused onto individual motors for 0.3–0.5 s, to bleach all GFP molecules within a motor while leaving as much as possible of the GFP elsewhere in the cell unbleached. Over time, motor spots reappeared, indicating that bleached GFP molecules in the motor—presumably accompanied by MotB—had been replaced by unbleached ones from elsewhere in the cell (Fig. 4b, c). FLIP experiments observed a decrease in spot brightness after selectively bleaching GFP outside motors, as bleached GFPs replaced unbleached ones in the motor. This served as a control for artifacts such as spontaneous recovery or late maturation of GFP fluorescence. Given the stable incorporation of MotB into stator units [16], the results demonstrated that stator units in the motor are constantly exchanging with a membrane pool of ~100 units, with a typical dwell time in a motor of only ~30s. We also estimated that each motor contains 11 ± 3 stator units (~22 ± 6 MotB-GFPs), consistent with the resurrection experiments.

Protein exchange was soon confirmed and extended to other components of the BFM in our lab and others [7, 39–42]. These observations of exchange in multiple genetically distinct components of the complex raised a number of questions. Is protein exchange a universal feature of large protein complexes? What functions does it serve? The replacement of defective subunits was one possible function, as was some kind of regulatory role. What is the range of timescales for protein exchange in different components and complexes? How can we reconcile protein exchange *in vivo* with the stability of purified complexes *in vitro*?

Stator unit exchange

Following the original measurements of stator exchange, it became clear that stator exchange dynamics are sensitive to multiple inputs. Stator units have reduced affinity for the motor when either the ion-motive force or the viscous load is reduced,

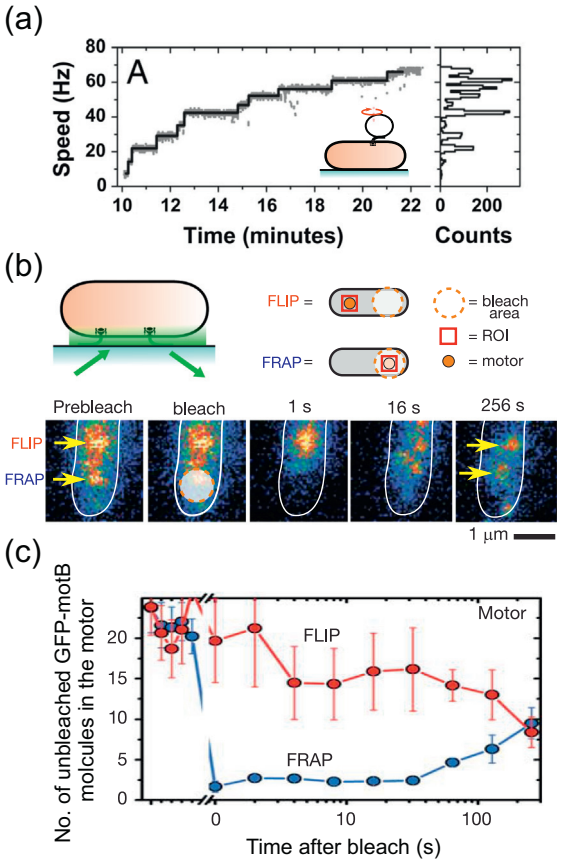

Fig. 4. Protein exchange in the flagellar stator. (a) Stepwise speed increments of an *E. coli* motor driving a polystyrene bead attached to a sheared filament stub, following the induction of stator proteins in a stator deletion background [34]. (b, c) FRAP and FLIP of single flagellar motors expressing GFP-MotB in *E. coli*, observed by TIRF microscopy [6]. In FRAP experiments, fluorescent spots reappeared within minutes at the locations of photobleached motors (b, lower spot; c, blue), in FLIP experiments, unbleached motor spots decreased in intensity over the same timescale after photobleaching of a separate part of the cell (b, upper spot; c, red). On average, spot intensities at long times after the bleaching pulse were the same in FRAP and FLIP, indicating complete mixing of bleached and unbleached molecules in the cell.

consistent with a mechanism in which binding of a stator unit to the cell wall is stabilized by force. Recent evidence indicates that the affinity of stator units for the motor also depends on other environmental factors and cellular signals. We consider that all these properties are functionally beneficial, hinting that stator unit exchange may be selected for—not an evolutionary accident.

Stator dependence on ion-motive force

The loss and subsequent stepwise resurrection of motor rotation following the transient removal of IMF, observed in *E. coli* expressing native H^+-driven stator units [43] or recombinant Na^+-driven homologs from *V. alginolyticus* [44], provided the first evidence that incorporation of stator units in the motor requires IMF. Direct observation followed in *V. alginolyticus*: fluorescently labeled stator component PomB delocalized from flagellar motors when sodium-motive force (SMF) was removed, when a sodium channel blocker was added, and in non-motile PomB mutants [45]—all interventions which remove the force between stator and cell wall. Using the light driven proton pump proteorhodopsin in combination with uncouplers of the proton-motive force (PMF) to allow for fast control of PMF in *E. coli* [46] (Fig. 5a), the incorporation of stator units in the motor following PMF removal was found to decay with a similar lifetime to that estimated in the original FRAP experiments [6].

Stator units conduct ions only when bound to the motor—when unbound, a "plug" domain blocks the channel [49], preventing leakage of ions across the membrane which would waste energy and potentially (for H^+-powered stators) disrupt the pH of the cytoplasm [50]. It is plausible that the loss of stators at low IMF serves to conserve energy at times when resources are limited, and the homeostasis of the cytoplasm is threatened.

Conversely, although the H^+-powered motor of *S.* Typhimurium is closely related to that of *E. coli* [51] and exhibits stator exchange in FRAP experiments [52], neither pore blocking mutations nor the removal of PMF were observed to disrupt stator protein localization to *S.* Typhimurium motors [53]. This suggests that IMF-dependent incorporation of stator units is not an inevitable consequence of stator protein exchange.

Stator mechanosensing

Two different experiments in 2013 demonstrated an effect of mechanical load on the number of stator units incorporated into *E. coli* motors, measured by the intensity of fluorescent motors containing GFP-labeled MotB and by stepwise speed changes [47, 54]. Following sudden increases of load caused either by cell tethering or attachment of 1-μm diameter polystyrene beads to filament stubs, the number of stator units increased from 1 to 2 to 6–10 over a few minutes [54]. Similarly, the steady-state number of stator units was found to increase with increasing viscous load and was maximal in motors stalled by attached magnetic beads [47] (Fig. 5b). Together with the decreased motor binding of paralyzed stator mutants [45, 55] and recent measurements of stator kinetics under rapidly varying load [56], this is consistent with a catch-bond model in which the bond between the *E. coli* stator unit and motor strengthens with applied force [55, 56]. This would confer sensitivity not just to load but also to ion-motive force, channel blockers, stator mutations, binding of

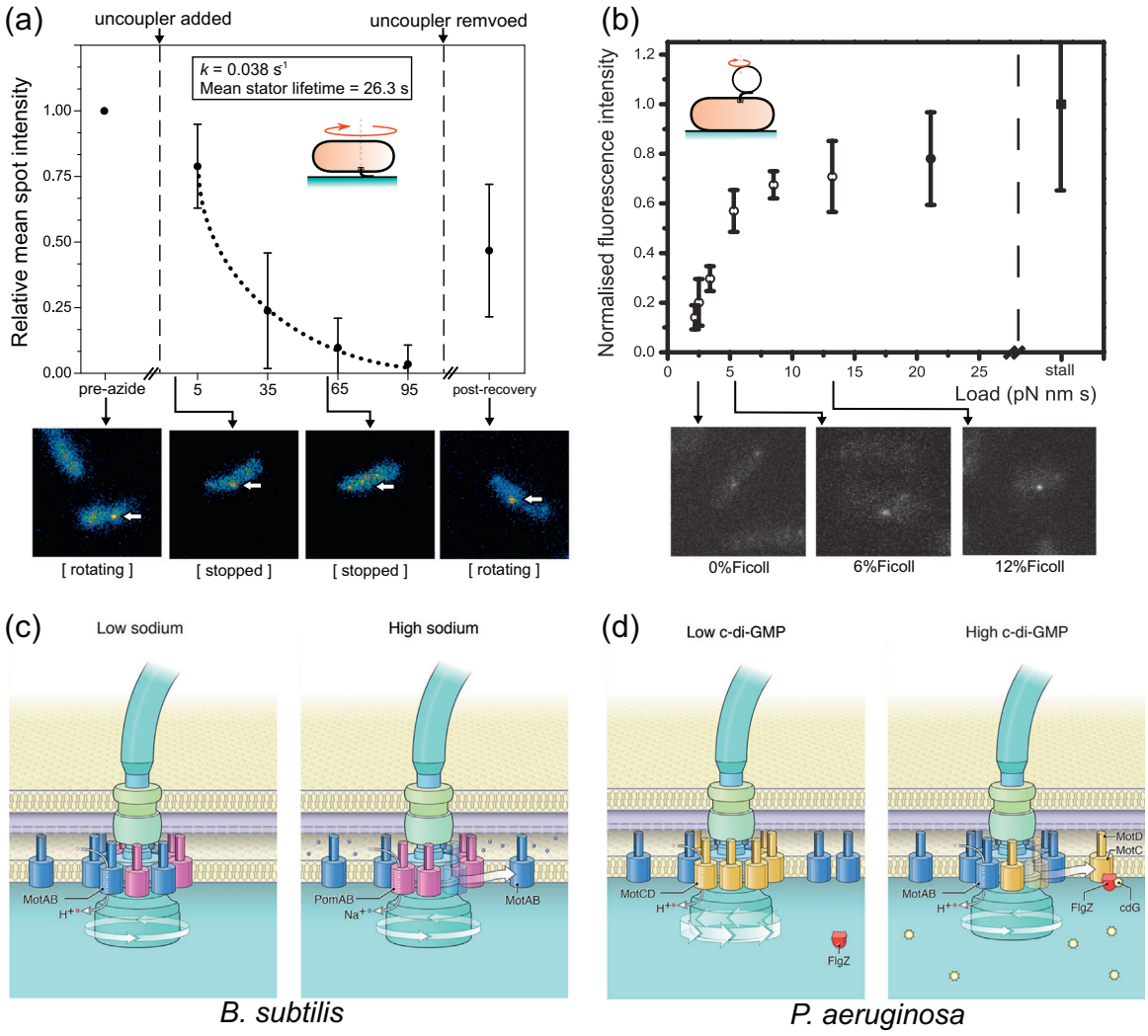

Fig. 5. Stator dynamics in BFMs. (a) Loss and subsequent restoration of GFP-MotB-labeled stator units in tethered *E. coli* following the removal and subsequent restoration of PMF by use of an uncoupler. The graph summarizes 11 experiments on separate cells. TIRF images show a time series from a single experiment [46]. (b) GFP-MotB-labeled stator unit incorporation in separate CCW-locked *E. coli* motors driving polystyrene beads in different viscous load conditions. The conditions shown are, in left-to-right order: 0.35-μm beads in 0%, 1%, 3%, 6%, 9% and 12% Ficoll (light circles), 1-μm beads with no Ficoll (filled circle) and magnetic beads stalled by an applied magnetic field (filled square). More than 15 motors were measured for each condition, except for stall where 5 motors were measured. Fluorescence images show three representative examples [47]. (c, d) Schematics of stator dynamics in *B. subtilis* and *P. aeruginosa*, respectively. Figure reproduced from Ref. [48].

regulatory proteins, and any other intervention which affects the force generated by a stator unit. The mechanism of mechanosensitivity may differ somewhat in other species—a *S. Typhimurium* stator mutation alters motor response to load without altering the number of stator units incorporated in the motor [57]. In many species the motor-binding protein FliL is also involved in load response [58–60], although this appears not to be the case in *E. coli* [54, 55].

Regardless of the mechanism, the disengagement of stator units at low load, as with IMF, is consistent with a biological function. At very low load—for example, in the nascent stage of filament assembly—a fully assembled stator ring would waste energy without propelling the cell. Furthermore, at lower loads, fewer stator units are required to achieve a given speed. There is disagreement as to whether extra stator units at very low load confer increased rotation speed, as they do at high load [61, 62]—they may just waste energy to no effect.

Stator isoform exchange and biochemical signaling

Many species encode a single set of BFM rotor proteins, but multiple isoforms of stator proteins [63]. Stator exchange in such systems was first studied in

Shewanella oneidensis MR-1, which expresses both Na^+- and H^+-powered stator units, the latter recently acquired by horizontal gene transfer [64] (Fig. 5c). Both isoforms function in the motor simultaneously and exchange competitively—H^+-powered stator units exchange faster in the presence of Na^+-powered stator units, and *vice versa* [65]. The proportion of Na^+-powered stator units in the motor is increased at high SMF, not through increased affinity of the Na^+-powered stator units but by reduced affinity of the H^+-powered units [65]. Competitively exchanging Na^+- and H^+-powered stator units are also found in the motor of *Bacillus subtilis* [66, 67]. As in *S. oneidensis* MR-1, the balance of isoforms responds to SMF, but the mechanism appears different: a peptidoglycan-binding domain in the Na^+-powered stator unit, which stabilizes binding to the motor, is only fully folded and active in the presence of Na^+ [68].

It is plausible that by tuning the balance of isoforms in this way, motors of both species use exchange to rapidly adapt, making use of whichever power source is the most abundant. However, similar tuning arises in *E. coli* motors assembled with both native H^+-powered stators and recombinant Na^+-powered stators, engineered by replacing most domains of the *E. coli* stator proteins with their homologs from *V. alginolyticus* [69]. Neither parent stator evolved to work in a dual-stator motor, although both are individually IMF responsive. It is curious that *S. oneidensis* MR-1 has apparently evolved to exploit competitive exchange by regulating the incorporation of one stator unit isoform through competition with the other.

Mechanosensing, like IMF, can regulate the balance of stator isoforms in dual-stator systems. In *B. subtilis*, incorporation of Na^+-powered stator units over H^+-powered competitors is promoted not only by high SMF, but by high load [70]. Interestingly, the polysaccharide Ficoll also promotes incorporation of Na^+-powered stator units, beyond the level that can be explained by the associated change in the viscous load external to the motor [70]. The mechanism of this is unclear—it may be that Ficoll impedes the motor directly, acting as an added load which affects stator incorporation *via* mechanosensing. As bacteria secrete polysaccharides during biofilm formation, polysaccharide sensing is likely to have a biological function. This has been demonstrated in *S.* Typhimurium, where motor inhibition by secreted polysaccharide contributes to motility regulation [71].

Motility regulation has also been studied in *Pseudomonas aeruginosa*, which has two stator unit isoforms—MotAB and MotCD. Both are H^+-powered, but only MotCD can produce rotation at high load—for example, swarming on a surface [72] or rotating a tethered cell. Upon tethering to a surface, *P. aeruginosa* rotate initially only if MotCD is present, and then rapidly stop only if MotAB is present, in response to a Cyclic adenosine monophosphate signal triggered by surface sensing [73, 74]. Similarly, at elevated levels of cyclic diguanylate (cyclic-di-GMP) the protein FlgZ destabilizes MotCD incorporation, leading to the loss of MotCD from the motor and the inhibition of swarming motility only in the presence of competing MotAB (Fig. 5d) [72, 75]. While exchange of stator units has not been measured directly in *P. aeruginosa*, this behavior is strongly indicative of competitive exchange as seen in *S. oneidensis* MR-1, used to facilitate regulatory control.

Cyclic-di-GMP, a secondary messenger associated with the transition to sessile lifestyle, also regulates motility *via* FlgZ homologs in a number of other species. In *B. subtilis*, FlgZ homolog MotI interacts with MotA and acts as a clutch—removing power from the motor, but not inhibiting its rotational diffusion [76]. This is consistent with reduced stator unit affinity for the motor. However, the FlgZ homolog in *E. coli* and *S.* Typhimurium (YcgR), both single-stator species, inhibits motility through a brake mechanism that impedes rotational diffusion of the motor and requires stator units to be expressed [71, 77, 78]. This suggests a different mechanism of action to FlgZ or MotI—one which does not require stator unit exchange, and indeed may act through binding to the C-ring [78] rather than the stator [77]. Similarly, *B. subtilis* possesses a second clutch protein in addition to MotI, EpsE, which acts through binding to the rotor rather than the stator [79].

While the biochemical details are unclear, there is evidence across many species that BFM mechanosensing is responsible for surface sensing which triggers lifestyle changes [48, 80]. Interestingly, the deletion of *P. aeruginosa* FlgZ alters the intracellular c-di-GMP level, suggesting that it may be responsible for bridging mechanosensor input and regulatory output [75]. Another stator-binding protein, DgcB, has been shown to perform such a role in *Caulobacter crescentus*—synthesizing c-di-GMP in response to motor–surface interaction [81]. An interesting possibility is that these pathways might be measuring the number of incorporated stator units as a proxy for load.

Stator exchange—perspective

The above evidence suggests that stator unit exchange is exploited to allow for rapid adaptation of motor function to changes in energy availability and output requirements. Competitive exchange of different stator unit isoforms is also exploited for adaptation and provides a handle for biochemical regulation. It is possible that exchange facilitates regulation in single-isoform systems also, and may constitute part of the readout pathway for motor mechanosensing.

However, stator exchange has been studied directly in relatively few species. In single-stator species,

stator exchange does not appear to be necessary for biochemical regulation, even if it is sometimes exploited to that end. In addition, while *E. coli* and *S.* Typhimurium stators are not resolvable *in situ* by cryo-ET, presumably because low viscous loads in sample preparation lead to stator dissociation, a number of other species have large periplasmic structures supporting well-defined and readily imaged rings of stator units (e.g., Fig. 2e, f) [24, 82, 83]. This raises the possibility that no stator exchange takes place in these species.

Rotor protein exchange

Repeating the 2006 stator FRAP experiments [6] with the C-ring component FliM [7] labeled with a yellow variant of GFP (FliM-YPet), we were surprised to find that it too was exchanging in functional motors—a result we had no reason to expect [7] (Fig. 6a). Typical dwell times were similar to stator units (~40s), and we measured a stoichiometry of 30 ± 6 FliM per motor, consistent with the rotational symmetry seen in EM structures [26]. FliM exchanged only in the presence of functional CheY, pointing to a role for FliM exchange in regulation of switching.

FliM exchange was also reported almost simultaneously by Fukuoka and co-workers [39], but with significant differences in reported kinetics. We attribute this to shortcomings in their experimental methodology, which we detail below in our discussion of FliG exchange. Subsequent studies have confirmed our initial measurements and elucidated a mechanism for motor adaptation (Fig. 7a) that relies on exchange. FliM exchange was shown to vary not with [CheY] *per se*, but with the rotation direction of the motor [42] (Fig. 7b). With fluorescently labeled FliM (FliM-eYFP), CCW-only motors in strains lacking CheY were ~30% brighter than motors made CW only by a mutation in FliG [42] (Fig. 7b, c). When CheY-P levels drop in wild-type strains, CCW bias increases, resulting in the recruitment of additional FliM to the ring, conferring greater sensitivity to CheY-P. This adaptive remodeling allows the ring to be extremely sensitive to changes in CheY-P levels on ~1 s time scales (Hill coefficient of ~20) [84], while adapting to longer-term changes in the average levels of CheY-P [41, 42, 85]. It also contributes significantly to the rapid (<10s) adaptation of the overall motor-chemosensory system to changes in the concentration of external chemoattractants or chemorepellents [86].

FRAP and stoichiometry experiments with FliN showed very similar dynamics to FliM [8] (Fig. 6b). Branch *et al.* [87] also found FliN dynamics to be very similar to FliM, except ~4 times slower. As their fluorescent protein fusion also slows functional adaptation of the motor, this is likely an artifact of labeling. Stoichiometry estimates were consistent with either 4:1 [88] or 3:1 [89, 90] FliN:FliM stoichiometry models, and together the data suggest that FliM and FliN may exchange as a 4:1 or 3:1 FliN:FliM unit. The structural details of FliM and FliN exchange remain unclear. Exchange measurements suggest the existence of separate stable and exchanging sub-populations of FliM/FliN, and that conversion between stable and exchanging states may be part of the mechanism for adaptation [7, 8, 42, 86]. The molecular mechanism for this has yet to be determined, although models have been proposed [91, 92] (Fig. 7d). In particular, it is unclear how the C-ring accommodates variation in the number of FliM/FliN units, or how this relates to the role of FliN in supporting the cytoplasmic export apparatus. Some models envision a fixed-diameter C-ring ring, with a fixed number of FliM/N binding sites [85], which can tolerate either gaps (Fig. 7d, right) or binding of "extra" FliM/N units not incorporated into the ring (Fig. 7d, left). Others suggest that the ring expands and contracts to accommodate changing FliM/FliN numbers [42, 91] (Fig. 7d, middle). Averaged EM

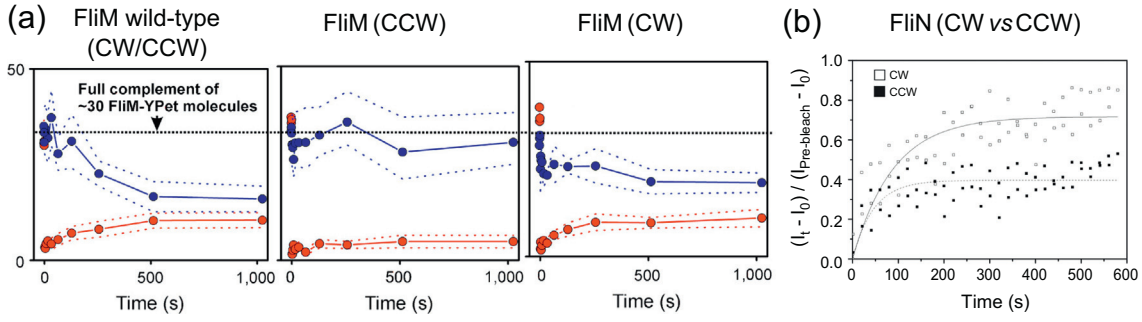

Fig. 6. Protein exchange in the flagellar rotor. (a) FLIP (blue) and FRAP (red) of fluorescently tagged FliM in tethered *E. coli*—traces averaged over 7–11 cells [7]. Non wild-type motors rotate CCW or CW as a result of CheY deletion or a CheY mutation mimicking phosphorylation, respectively. The CCW cells lacking CheY show much reduced exchange. Note that FLIP and FRAP curves do not converge, unlike for MotB (Fig. 2b), indicating a fraction of FliM in the motor which is not being replaced by unbleached molecules (FRAP) or bleached molecules (FLIP), and therefore not exchanging. (b) FRAP measurement of fluorescently tagged FliN, equivalent to the red traces in panes 2 and 3 of (a) [8]. Recovery dynamics are very similar to FliM.

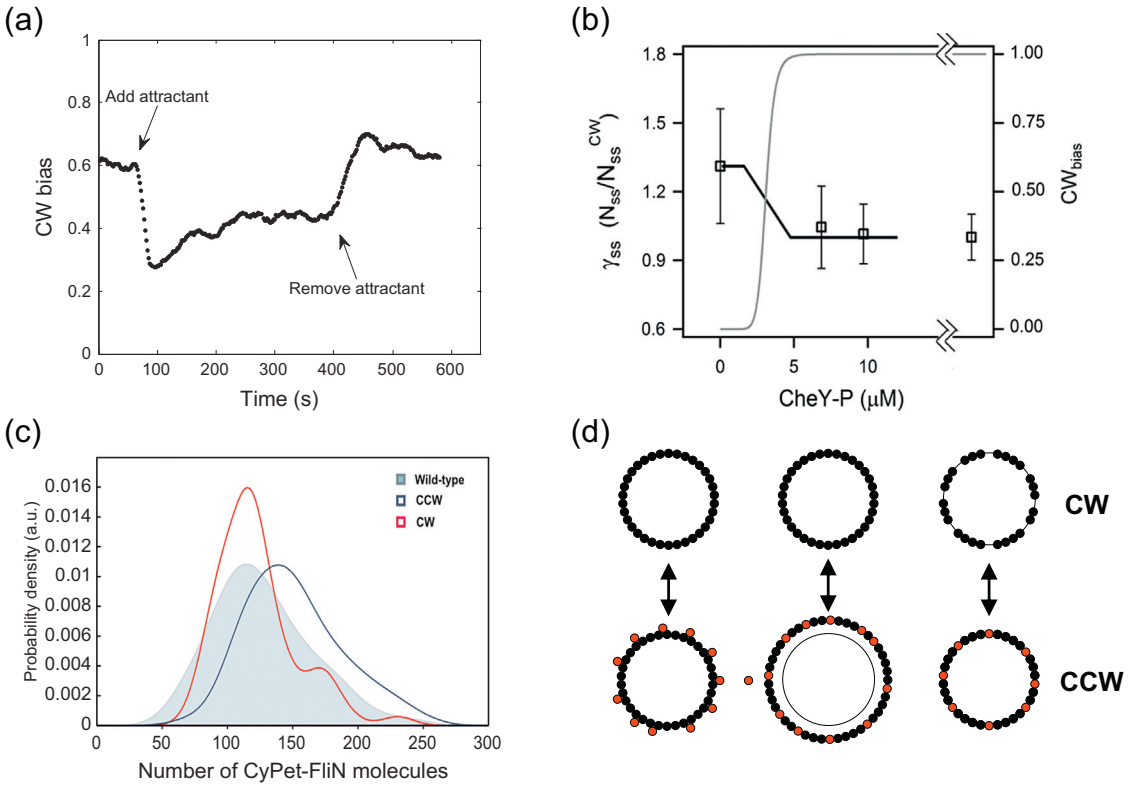

Fig. 7. (a) Motor rotation response in mutant *E. coli* cells lacking any adaptation in their chemoreceptors, such that average CheY-P levels are expected to stay constant for a given level of attractant. Addition of chemoattractant causes a decrease in average CheY-P concentration, which initially leads to a decrease in CW bias: the proportion of time the motor spends rotating CW. Over time, the motor adapts to the new CheY-P concentration, increasing its sensitivity such that CheY-P triggers CW rotation more readily—the CW bias partially recovers toward its starting level. The partial recovery of rotation bias indicates adaptation of the motor itself to changed CheY-P levels [84]. (b) Variable quantity of motor-bound FliM (left-hand axis) measured in *E. coli via* fluorescence intensity. Motors with wild-type switching behavior (3 left-most data points, black curve) have decreased FliM incorporation at elevated CheY concentration. FliM incorporation in mutant CW-locked motors in the absence of CheY (right-most point) is also low, demonstrating that FliM incorporation varies with rotation bias rather than CheY-P concentration *per se*, consistent with the wild-type dependence of rotation bias on CheY concentration (right-hand axis, gray curve). [42]. (c) Variation of FliN stoichiometry with rotation direction, measured by fluorescence intensity. Stoichiometries are an estimate based on comparison with fluorescently labeled stators as a control of known stoichiometry [8]. (d) Possible models of how the C-ring could accommodate the observed variation in FliM/N numbers. Extra units that are present in CCW motors are illustrated in red.

structures of purified CW-locked [27] and wild-type [22] motors show ~34-fold C-ring symmetry and the matching ring diameter, respectively. Removal of circulating FliM/N during purification might be expected to act as a ratchet for protein exchange—FliM/N units that leave the motor cannot be replaced, driving the ring toward a minimal stable size. EM reconstructions of motors crosslinked *in vivo* to preserve native CW and CCW compositions could in principle reveal this effect. If so, the symmetry of the average EM image would reflect the minimal stable ring in the expanding ring model, or the unvarying underlying ring of binding sites in the "gaps" and "extras" models, assuming gaps and extras are unresolved by EM. On the other hand, if protein exchange is frozen by purification, variable ring symmetries from 31-to 38-fold seen in overproduced wild-type rotors [27, 28] might reflect the natural range in the variable ring model. The gaps model predicts ~30% reduced density in the corresponding part of the averaged CW rotor reconstruction, compared to predictions based on the molecular masses of the components. This should be detectable, but so far has not been reported.

Exchange in a number of additional components has also been investigated (Fig. 2a). FliI, a component of the export apparatus homologous to the α and β units of F_1-ATPase, was shown to exchange several times per minute [93]. This does not appear to be driven by ATP hydrolysis and is too slow to account for delivery of export substrates to the motor. FliI is not essential for export, but powers a rotary "ignition key"; hydrolyzing ATP only at a slow rate, but somehow increasing the efficiency of export [94, 95]. The role of

exchange is unknown. Exchange on a timescale of minutes has also been observed in export apparatus protein FlhA [40, 96], which forms both the transmembrane ion pore responsible for powering protein export, and part of the nonameric cytoplasmic gate which controls the substrate-specificity of export through conformational changes. As for FliI, the role of exchange is unknown.

Conversely, reports exist that neither FliG [39] nor FliF [40] exchange on timescales of ~2 h and ~10 min. respectively. However, the use of long wide-field exposures for the photobleach phase of FRAP in the former study [39] resulted in the bleaching of large but unquantified fractions of the entire cellular GFP population, making these reports unreliable. For example, the exchange of FliN was estimated to occur with characteristic times of 20 min [39] rather than ~40 s measured with fast and tightly focused photobleaching illumination [8]. Anomalies in FliN FRAP—including further reductions rather than recovery of fluorescence after shorter bleaching phases and the inability to quantify FliM exchange, presumably because of the very low signal/noise ratios after excessive photobleaching—further illustrate the unreliability of these measurements. FliG copy numbers are expected to be similar to FliM, 3–4 times lower than FliN, and so the possibility remains that FliG exchange occurs but was missed in these experiments.

Finally, protein exchange may have a role in assembly of the BFM rotor. A number of *in vivo* fluorescence studies visualized putative pre-assembly complexes in *E. coli* [7, 40, 97]—we measured these to contain ~20 fluorescently tagged FliM molecules, undergoing negligible exchange on minute timescales [7]. A study quantifying the incorporation of several fluorescently tagged BFM components into complexes, and the inter-dependencies of various components found results consistent with a model of sequential but cooperative assembly, whereby incorporation of a component class is incomplete until stabilized by the binding of downstream components [40]. The relation of exchange to these observations is an interesting avenue for future study. It may be possible that some components are stable in pre-assembly intermediates but exchanging in the complete motor, and *vice versa*. There is also evidence that FliG, FliF, and FliM form a variety of pre-assembly complexes in the cytoplasm [8, 98]—the influence this might have on FliM/N exchange is unclear.

In summary, there is clear evidence that subcomplexes of FliM and FliN exchange in *E. coli* motors, fine-tuning C-ring function through variable subunit stoichiometry. Exchange is also observed in FlhA and FliI, but the functional benefit (if any) has yet to be uncovered. Finally, there is no exchange in FliF and prospectively not in FliG, at least over short timescales (10s of minutes to hours). This supports the notion of a stable central core to the motor on which less stable peripheral components are mounted, and suggests that exchange is not a general requirement for components of a large complex.

Protein exchange in the injectisome

The "injectisome" is a needle-like protein export complex used by many pathogenic bacteria to deliver toxins, which shares a common evolutionary ancestry, significant homology and structural similarity with the BFM [25, 99]. Export in both complexes is highly regulated, and driven by a type III secretion system (T3SS). The injectisome also possesses a putative C-ring, consisting of YscQ (here we use component names from *Yersinia enterocolitica*, other species have different names for essentially the same genes) which is moderately homologous to FliM, and $YscQ_C$—the product of an internal translation start site comprising the C-terminal third of YscQ, which is highly homologous to FliN [25]. Both are required for injectisome assembly and share similar interactions to their BFM counterparts [25, 89, 90].

Exchange has been studied in a limited number of injectisome components. In *Y. enterocolitica*, FRAP over ~10 min showed negligible exchange for both YscV, a homolog of exchanging BFM component FlhA (Fig. 2a), and YscC—a secretin with homologs in other secretion complexes, expected to form a stable ring in the outer membrane [25, 100]. YscQ, however, was shown to exchange over minute timescales, similar to the homologous FliM/N. Exchange was faster in conditions promoting active secretion, but faster still in the presence of a catalytically inactive mutant of the T3SS ATPase, which cannot support secretion [100]. Although this hints at a functional role for YscQ exchange, none has been elucidated so far—as with BFM FliI, exchange rates were too slow to correspond to delivery of export substrates.

In vivo fluorescence correlation spectroscopy, used to identify co-diffusion and measure apparent diffusion coefficients of labeled proteins, has revealed that YscQ, YscL, YscN and YscK interact in the cytoplasm, forming pre-assembly complexes reminiscent of those reported in the BFM. It is unknown whether they exchange as a unit. Extracellular Ca^{2+} concentration, which controls injectisome activation, also affects the interaction strengths within these complexes [101]. As a change in injectisome C ring structure is associated with activation [102], this raises the possibility that activation is mediated through a change in interaction strengths that alters exchange dynamics and consequently structure.

Summary

Many proteins in the BFM and Injectisome, but not all, are exchanging on ~minute timescales. Compositional flexibility is far in excess of what we might have imagined a few decades ago—both

the stoichiometry of exchanging components and the balance between competing component isoforms are dynamic. Furthermore, the more we study exchanging components, the more we find that exchange underlies behaviors with plausible biological function. Many of those functions could conceivably be served by mechanisms not requiring exchange.

This leads us to the hypothesis that exchange is not a general requirement of components of a large protein complex, but is nevertheless common and readily exploited by evolution to serve diverse functions. To investigate whether this may be true universally, and not just a quirk of the BFM/Injectisome, we must look to studies in other complexes.

Protein exchange in other well-studied complexes

Figure 8 and Table S1 summarize all the examples of subunit exchange as we have defined it that we have found in the literature. From these examples, it is difficult to conclude much about the *prevalence* of subunit exchange in general, as relatively few complexes have been well studied, and negative results are not well-reported. However, we can still draw some simple conclusions from these examples. First, subunit exchange appears to be common to all domains of life, although there are no *in vivo* observations in archaea probably because it has been difficult to engineer functional fluorescent fusion proteins in archaea [103]—although technical advances may overcome this obstacle in the near future [104]. Second, while many published FRAP measurements (including our own) are too short-lived to measure exchange on timescales longer than a few minutes, a handful of longer observations with FRAP and other techniques demonstrate that there is a very broad range of timescales over which exchange can occur, even within the same complex. This is well illustrated by the nuclear pore complex (NPC), where a systematic FRAP study measured component lifetimes ranging from seconds to hours—the entire range accessible by FRAP [38]. Protein exchange on longer timescales was probed indirectly by isotopic labeling to measure the turnover rates of thousands of proteins, including NPC components, in rat neurons [105]. Rats were fed on a diet containing exclusively ^{15}N until the sixth week of birth. Mass spectrometry was later used to identify, on a protein-by-protein basis, the fraction of remaining proteins that had been synthesized during the ^{15}N diet. While some NPC components had been completely replaced after 6 months, the most stable comprised 25% ^{15}N even after a year, despite robust translation of new protein. NPCs have been observed to disassemble entirely only during cell division, and

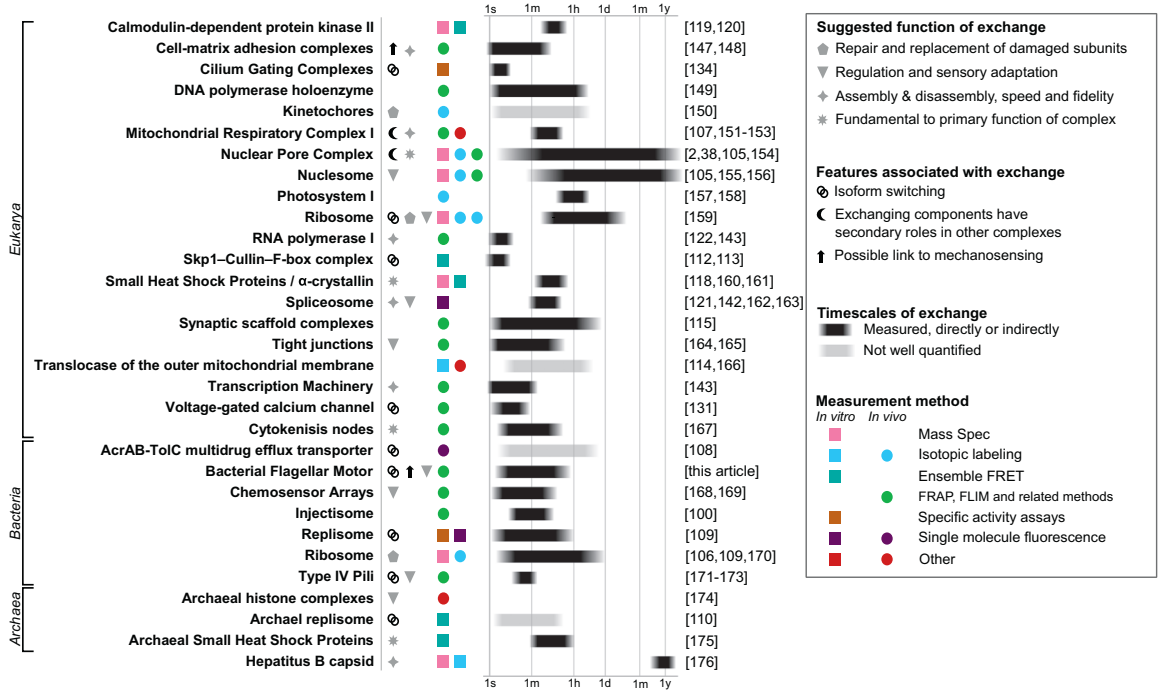

Fig. 8. A summary of examples of subunit exchange that we have found in the literature. More detailed descriptions can be found in Table S1, the main text and references [147–176]. We only list hetero-complexes here, except for one or two particularly interesting examples. Indications of timescale are approximate, and in most cases exchange over long timescales has not been characterized. We list functions of exchange that have been suggested in the literature, but note that in many cases, they are speculative.

this cell type was selected for its minimal division rates. Some NPC components were found to be extremely long lived, an observation best explained by protection from digestion by stable sequestration in long-lived NPCs [105]. However, even these most stable of proteins do turn over. It is possible that every component of the NPC is exchanging on a timescale shorter than the lifetime of the complex.

Replacement of damaged proteins

Isotopic labeling of NPC components [105] showed that complexes purified from old neurons were comparatively deficient in those components known to be the most-long lived, but not those which are exchanging more often. This was consistent with degradation or loss of the long-lived components from their complexes. This has been linked to a breakdown in the integrity of the nuclear envelope, and (along with other long-lived proteins) identified as a possible cause of aging [2]. It has been suggested that the NPCs are so long-lived because they cannot be disassembled without disrupting nuclear integrity, and that their long-lived components are sufficiently essential to NPC stability to prevent exchange for the same reason [2]. The rarity of such long-lived components even within long-lived complexes may conceivably reflect a strong evolutionary pressure for components in long-lived complexes to exchange, in all but the most restricting of circumstances. There is limited evidence suggesting that the need for repair (replacement) may contribute significantly to such a pressure. In the ribosome, the *in vitro* activity of damaged complexes can be partially restored through subunit exchange with a pool of spare parts, and there is a strong overlap between the subset of components known to exchange, and those which are most damage-prone [106]. Subunit exchange in mitochondrial respiratory complex I has also been suggested to allow for repair of oxidative damage [107].

Specific functions

Some of the examples in Fig. 8 also highlight possible functions of protein exchange. Multiple examples show competitive exchange between functionally different isoforms of the same subunit, similar to the dual-stator systems in the BFM. The bacterial AcrAB–TolC efflux transporter comprises an outer-membrane channel, and competitively exchanging inner-membrane transporters with specificity for different substrates. Presence of a substrate stabilizes incorporation of its corresponding transporter [108]. Likewise, exchange of DNA polymerases in bacterial replisomes [109] (and their archaeal [110] and eukaryotic [111] relatives) allows the transient use of specialized polymerases to overcome lesions in the DNA template. The Skp, Cullin, F-box containing (SCF) complex, responsible for ubiquitination of substrate proteins, rapidly cycles different adapter units (F-boxes) with specificity for different substrates. When an adapter is found with its substrate present, cycling halts to allow for ubiquitination [112, 113].

Isoform switching is not the only kind of heterogeneity enabled by exchange. Some complexes exist in multiple but well-defined isoforms, such as the translocase of the outer mitochondrial membrane (TOM) complex, where subunit exchange appears to facilitate cycling between a complete complex with a trimer pore, and a smaller complex with a dimer-pore, and presumably altered activity [114]. Others, like the BFM, have continuously variable subunit stoichiometry; synaptic scaffolds [115] and the NPC [116, 117] are examples. In both cases, early indications are that such stoichiometric changes represent functional changes. In the family of small heat shock proteins and α-crystallin, extreme heterogeneity facilitated by subunit exchange is thought to be central to their function as chaperones [118].

Exchange may facilitate information transfer between complexes. In homo-oligomeric rings of calmodulin-dependent protein kinase II, elevated Ca^{2+} levels activate the kinase activity of subunits which subsequently phosphorylate other subunits in the complex. *In vitro*, this activation also opens the ring, allowing for the exchange of activated subunits which can then incorporate into and activate other complexes. *In vivo*, this may increase sensitivity to Ca^{2+} and prolong activation after Ca^{2+} drop—a type of molecular memory [119, 120]. Exchange may also be important not just for the steady-state functioning of complexes but also for their assembly. In the spliceosome and RNA polymerase I, for example, subunit exchange is a manifestation of an assembly regime where each step is reversible [121, 122]. This potentially increases the fidelity of decision making. In the case of the spliceosome, for example, it is thought to facilitate regulation of splicing decisions throughout the assembly pathway. Understanding of that system has benefited from the recent development of single-molecule pulldown assays, in which target proteins in whole-cell extracts are bound to coverslip surfaces *via* antibodies or known binding partners soon after lysis. Complexed binding partners bound to the surface *via* the target protein may be visualized either by genetic fusion to fluorescent proteins, or tags which can be rapidly dye-labeled post-lysis. The speed of this method allows the single-molecule characterization of interactions too weak or transient to survive purification of the complex by more traditional methods [123]. We anticipate such techniques becoming increasingly important for the study of protein exchange generally.

Broader proteome-level investigations

While the relatively detailed studies described above are instructive, they are limited in their ability to describe

extent of protein exchange across an entire proteome. The last 10–15 years have seen a rapid growth in high-throughput proteomic studies, particularly in eukaryotes. While none to date directly address exchange, many measure properties which we expect to be associated with exchange—weak protein–protein interactions strengths in complexes, for example [124]. While such measurements cannot directly imply exchange, it is instructive to ask whether they are at least compatible with the hypothesis that exchange is widespread. Furthermore, if exchange really is widespread, high-throughput studies may offer some insight into possible functions (if any) that it might serve and phenomena which it might be associated with.

When the abundances of protein complex components are measured, a substantial and clearly differentiable minority of components have expression levels which vary significantly with cell type or state (often both), relative to their partners in a complex [125–128]. These can be described as an "attachment" group of proteins, as opposed to a "core" group which have more uniform expression, and are more likely to be transcriptionally co-regulated [128]. Proteins in the attachment group are more likely to be variant isoforms of other proteins in the complex, and it is common that elevated expression of one isoform corresponds with decreased expression of another [125]. This is mirrored by the evolutionary history of complexes in which typically a core group of components are rarely lost or gained while other more peripheral components are lost or gained readily [129]. This is also consistent with the composition of co-purified complexes analyzed with mass spectroscopy—often, multiple isoforms a complex are found, sharing a core group of proteins but differing in attachment proteins [128].

Given that many known examples of exchange involve isoform switching (see previous section and Fig. 8), it is interesting to consider whether the attachment proteins described above, particularly when consisting of mutually exclusive isoforms, may be commonly associated with exchange. There are certainly individual examples which suit this model—voltage-gated Ca^{2+} channels, for example, exist in complex with various mutually exclusive regulatory β-subunits, typically associated with different cell types [130]. Recent FRAP experiments show that at least some of these β-subunits, though not all, are exchanging [131]. High-throughput studies offer limited evidence, however, that this is the case more generally. Circumstantial evidence suggests that increased digestion rates of component proteins not sequestered in a complex may contribute significantly to the variation in relative abundances of components described above, much of which cannot be explained by translational or transcriptional regulation [125]. This would tend to argue against widespread exchange, which would sequester components only transiently. Conversely, however, attachment proteins do tend to have less hydrophobic binding interfaces, and so prospectively weaker interactions [125]. Indeed, high-throughput quantification of protein–protein interactions strengths demonstrates that most interactions in the proteome are weak [124]. This is also consistent with evidence of protein dynamism more generally—high-throughput microscopy with a library of GFP-tagged proteins in yeast demonstrated recently that the localization and abundance of many proteins (complexed and not) is highly responsive to stress conditions, significantly beyond the extent that can be explained by transcriptional or translational regulation [132]. Predominance of weak interactions and post-translational dynamism would tend to point to a widespread role for exchange. More tenuously, the subunit stoichiometries of well-studied complexes correlate poorly with relative expression levels [127]—while this variation may compensate for variation in interaction strengths or assembly kinetics [127], it is also reminiscent of what is seen with exchanging subunits in the BFM, where, for example, the pool of freely diffusing stator complexes is in a 10-fold excess over motor-incorporated stators, and thus appears to have anomalously high expression [6, 127].

It is also interesting to note that an estimated 8%–30% of proteins are associated with multiple complexes [127, 128, 133]. Such proteins may perform similar roles in broadly homologous complexes, or be involved in as signaling between complexes (as for CheY and the BFM), shared regulation, or a number of other functions [127, 133]. There are also widespread examples where a protein serves apparently unrelated roles in different complexes—various NPC components, for example, have secondary roles in cilial gating zone complexes [134], gene regulation, chromatin remodeling, mitotic control [135], and possibly in DNA repair, signaling and RNA processing [136]. Figure 8 highlights a number of examples where such multi-functional proteins are known to exchange. It is possible that exchange may be associated with this promiscuity, either because the exchange of shared proteins between complexes is functionally useful or because the promiscuity makes it more difficult to evolve strong binding interactions.

While much of the above is consistent with a widespread prevalence of exchange, it sits uneasily with the growing evidence that some components of protein complexes interact with their binding partners during or soon after translation, both in prokarya and in eukarya [137–139]. Co-translational assembly, like co-translational folding, presumably minimizes the opportunity for proteins to form unwanted or non-specific interactions. While other mechanisms exist to minimize unwanted strong interactions occurring out of context [140], there is evidence of significant evolutionary pressure for co-translational assembly. In prokarya, for example, there is evolutionary pressure for interacting components

to be co-translated on the same operon [141]. This is not obviously compatible with exchange, or consonant with post-translational flexibility. It is conceivable that co-translationally assembled components are those most prone to unwanted interactions, and the least likely to exchange.

In summary, while high-throughput proteomics data do not prove the hypothesis of widespread protein exchange, in general they are compatible with it. It is easy to imagine a role for exchange in a highly dynamic proteome where weak interactions dominate, complexes are compositionally variable and subunits are differentially regulated, often shared between complexes and often competing with paralogs. Furthermore, the studies above highlight the possibility that exchange is linked to "accessory" proteins, which may exhibit competition between isoforms, or functional roles in multiple complexes.

Perspective

All the evidence above suggests that, while not ubiquitous, protein exchange *may* be very widespread. The evidence, however, remains circumstantial, until high-throughput techniques can be brought directly to bear on questions of exchange. We speculate that high-throughput predictors of exchange might be uncovered by cross-correlation between existing genomic and proteomic databases and detailed measurements of protein exchange such as those that have been made in the BFM and the NPC. It remains unclear whether the extent and nature of exchange varies between the domains of life—in archaea particularly, we know very little.

It also remains unclear whether the prevalence of exchange is merely a consequence of an unrelated evolutionary preference for weak interactions, or whether exchange confers functional benefits that may contribute to that evolutionary preference. We hypothesize that the well-studied exchange behaviors in the BFM stator and motor confer multiple functional benefits. However, while we have highlighted many examples of other complexes where exchange may have been exploited to serve specific roles in regulation and adaptation, it is not clear how often this is the case. We can speculate as to what other pressures and constraints relate to exchange—for instance, how strong is the pressure for components to exchange as a mechanism for the removal and replacement of old and damaged subunits? The relation of exchange to both assembly dynamics and evolutionary dynamics also warrants closer attention. Might protein exchange during assembly serve a kinetic proofreading function, increasing fidelity of decisions on how and whether or not to finish constructing a complex, as has been suggested for the spliceosome [142] and RNA polymerase [143]? And in the "core plus attachments" picture of protein complexes, circumstantial evidence suggests that cores, which are best conserved between organisms, are also the most stable and well defined. Do evolutionarily more recently acquired subunits tend to exchange more?

Given the possibility that protein exchange is widespread, there are implications for the way we study and understand protein complexes. We notice that in many scientific fields, including our own, complexes initially assumed to be stable and homogeneous turn out not to be. In a number of these cases, *in vitro* experiments gave a false impression of stability—in systems, for example, where subunits are stable in the absence of competing subunits in solution [109], or where the presence of certain co-factors can massively change exchange rates [112]. For many scientists, practical considerations lead to the default selection of conditions *in vitro* for optimized stability. In systems such as the BFM, we still have yet to understand why purified complexes imaged by EM fail to reflect the structural heterogeneity we see *in vivo*. Although *in vitro* techniques are incredibly powerful, these examples should be cautionary.

Meanwhile, *in vivo* measurements are still difficult. While FRAP has been often used, it is only possible with complexes which remain roughly stationary, and requires the construction of fluorescent protein fusions which can impair function, induce oligomerization or otherwise alter native interactions [144, 145]. FRAP on individual complexes remains rare, being both technically challenging and amenable to an even smaller subset of complexes (individual complexes must be, for example, well separated). While the clear conclusion from examples such as the NPC is that exchange can occur over a very broad continuum of timescales, FRAP experiments have been limited to hours, and we note that most studies (our own included) observe for much shorter timescales. Although longer observations are possible in principle, the technical challenges and resource requirements are considerable.

Bridging the gap between *in vivo* and *in vitro* measurement, single-molecule pull-down methods provide new opportunities for the study of exchange, particularly in mobile or densely packed complexes not amenable to FRAP *in vivo* [123]. The rapid developments in electron microscope technology point to a future where compositional heterogeneities in complexes may be resolvable *in situ* with cryo-ET [4]. The same developments in electron microscope technology are also likely to assist the *in vitro* study of compositional heterogeneity, along with the continuing development of mass spectrometry techniques, which have already been applied to the study of exchange in a number of complexes [119, 146].

Historically, we have tended to group proteins into well-defined complexes (assumed stable) and proteins that we know interact transiently with the complex. In the BFM, for example, we consider FliM as part of the motor, but not its binding partner CheY or the export apparatus that has not yet been co-purified with the BFM. Mass proteomics on the other hand, due to a comparative lack of detailed data, tends necessarily just to group proteins by their interactions with each other. In this view, proteins like CheY are no less a part of the complex than proteins like FliM. With the discovery that protein exchange occurs on a continuum of timescales, perhaps it is necessary to replace a binary distinction between stable complexes and transient binding partners with a description of the lifetime and exchange dynamics of each individual protein associated with a complex. We must abandon any natural inclination to assume that complexes are stable and homogeneous, and we should be cautious about results which appear to imply stability. Rather, we should assume that all components are likely to exchange on *some* timescale. This constitutes a reminder of the fundamentals of biology taught in undergraduate courses, where biochemistry is described in kinetic terms—binding and unbinding, association and dissociation, on and off rates—which can be forgotten in the face of the powerful but static pictures that are presented by modern structural biology. Protein exchange is here to stay, and compositional dynamics and heterogeneity need to be considered in any satisfactory model of how a large protein complex works.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jmb.2018.06.039.

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Abbreviations used:
EM, electron microscopy; ET, electron tomography; AFM, atomic force microscopy; BFM, bacterial flagellar motor; IMF, ion motive force; PMF, proton-motive force; SMF, sodium-motive force; GFP, green fluorescent protein; FRAP, fluorescence recovery after photobleaching; FLIM, fluorescence loss in photobleaching; TIRF, total internal reflection fluorescence; CW, clockwise; CCW, counterclockwise; T3SS, type III secretion system; NPC, nuclear pore complex.

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