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In vitro and *in vivo* single-molecule fluorescence imaging of ribosome-catalyzed protein synthesis

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Combined with the availability of highly purified, fluorescently labeled *in vitro* translation systems, the advent of single-molecule fluorescence imaging has ushered in a new era in high-resolution mechanistic studies of ribosome-catalyzed protein synthesis, or translation. Together with ensemble biochemical investigations of translation and structural studies of functional ribosomal complexes, *in vitro* single-molecule fluorescence imaging of protein synthesis is providing unique mechanistic insight into this fundamental biological process. More recently, rapidly evolving breakthroughs in fluorescence-based molecular imaging in live cells with sub-diffraction-limit spatial resolution and ever-increasing temporal resolution provide great promise for conducting mechanistic studies of translation and its regulation in living cells. Here we review the remarkable recent progress that has been made in these fields, highlight important mechanistic insights that have been gleaned from these studies thus far, and discuss what we envision lies ahead as these approaches continue to evolve and expand to address increasingly complex mechanistic and regulatory aspects of translation.

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Introduction

The ribosome is the universally conserved, two-subunit ribonucleoprotein ribozyme that synthesizes proteins by sequentially incorporating aminoacyl-transfer RNA (aa-tRNA) substrates in the order specified by the codon sequence of a messenger RNA (mRNA) template, a process termed translation (Figure 1) [1]. Over the past eight years, single-molecule fluorescence imaging has significantly expanded our mechanistic understanding of translation. We begin this article by briefly reviewing the prolific body of work that has emerged from single-molecule *in vitro* fluorescence studies of translation. Using a top-down approach, we open with a discussion of studies in which the overall rate of protein synthesis by single ribosomes has been measured and conclude with a synopsis of the numerous studies in which partial reactions within the translation pathway have been kinetically dissected with single-molecule resolution. We follow this by highlighting a number of exciting recent reports in which protein synthesis and ribosomes have been imaged in living cells using cutting-edge *in vivo* single-molecule fluorescence imaging approaches. Collectively, these advances in fluorescence imaging of translation are enabling researchers to address mechanistic questions that have remained difficult or impossible to address using ensemble biochemical approaches.

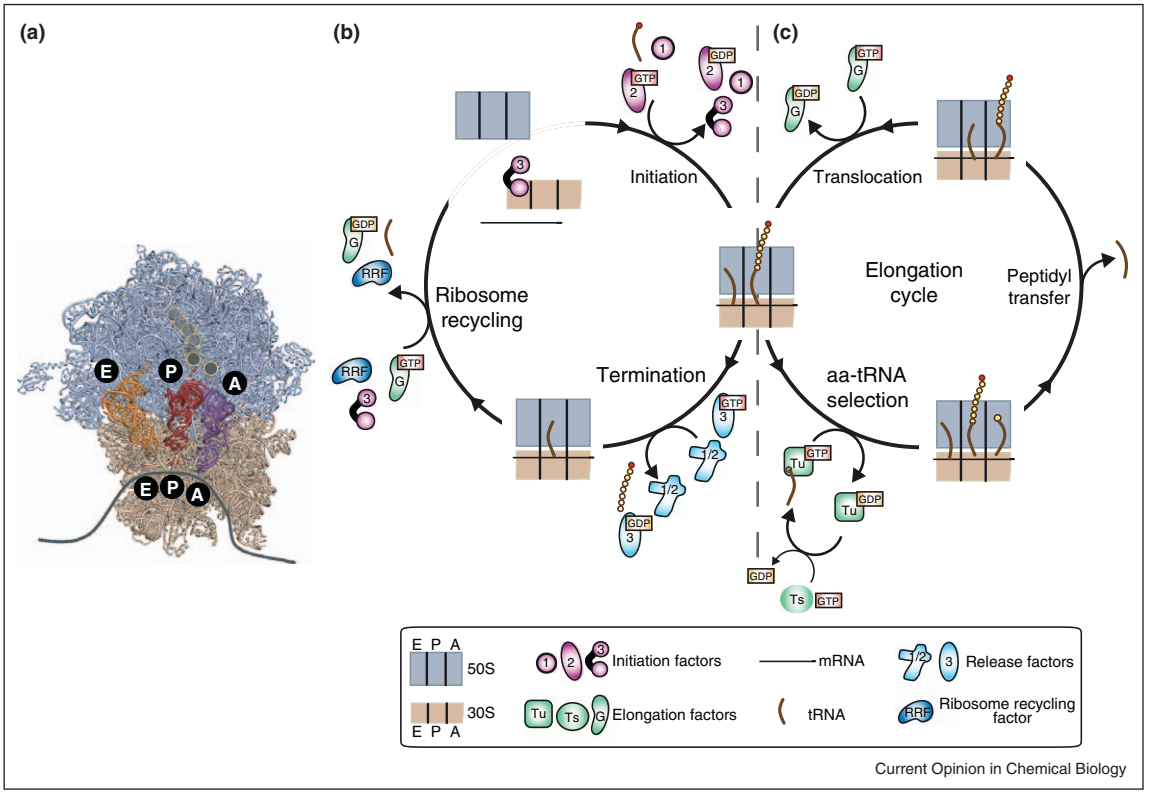
In vitro imaging of ribosome-catalyzed protein synthesis

One of the most important technologies enabling *in vitro* fluorescence imaging of translation at the single-molecule level is the ability to reconstitute the entire protein synthesis reaction in a test tube using purified translation components [2]. By replacing wild-type translation components with recombinantly expressed and fluorescently labeled variants that retain full biochemical activity [3], several research groups have been able to use various fluorescence imaging approaches to investigate the molecular mechanism of protein synthesis with single-molecule resolution (reviewed recently in [4,5]). These studies have made use of epifluorescence [6,7], confocal fluorescence [8], and total internal reflection fluorescence (TIRF) [9,10] microscopy approaches, or slight variations thereof, to image single ribosomes or ribosomal complexes and address mechanistic questions ranging from the rate at which single ribosomes translate single mRNAs to the roles that ribosome, tRNA, and translation factor conformational dynamics play in driving and regulating protein synthesis.

Measuring the rate of protein synthesis by single ribosomes

The first demonstration that protein synthesis could be observed with single-ribosome resolution came from Cooperman, Goldman, and co-workers (Figure 2a and b) [11•]. In this study, ribosomal elongation complexes carrying a 3′-biotinylated mRNA encoding a polyphenylalanine polypeptide were immobilized via non-specific adsorption to mica. The mRNA 3′-end was subsequently labeled using a 0.2 μm diameter, neutravidin-derivatized, and fluorescently labeled polystyrene bead. Epifluorescence

Figure 1

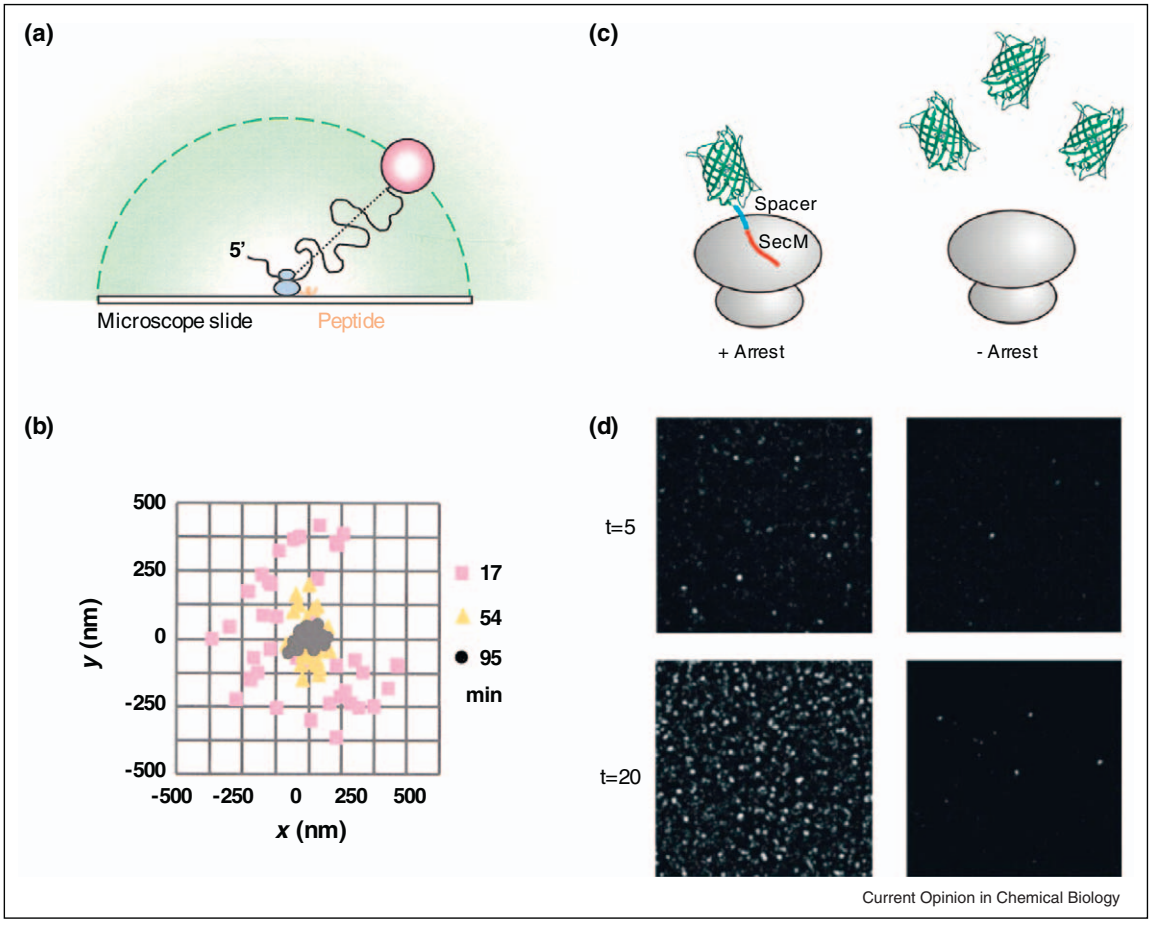


Ribosome structure and the translation process. **(a)** The X-ray crystallographic structure of a prokaryotic ribosomal elongation complex [12]. The large ribosomal subunit is depicted in blue, the small subunit is shown in tan, and the mRNA is cartooned in gray. The ribosome contains binding sites for aa-tRNA (purple), peptidyl-tRNA (red), and deacylated tRNA (orange) which span the two subunits and are designated as the A, P, and E sites, respectively. **(b-c)** The prokaryotic translation process. During the initiation stage of protein synthesis, a ribosomal initiation complex is assembled in which an initiator fMet-tRNA^{fMet} and the start codon of the mRNA to be translated are positioned into the P site. Initiation complexes then proceed into the elongation stage of protein synthesis, during which elongating ribosomes cycle through three major steps as each amino acid is added to the nascent polypeptide chain: (i) aa-tRNA selection; (ii) peptide bond formation; and (iii) translocation. The elongation cycle is repeated at each codon until a stop codon is translocated into the A site, an event that triggers the termination stage of protein synthesis and the release of the newly synthesized protein from the ribosome. The resulting ribosomal post-termination complex then enters the ribosome recycling stage of protein synthesis during which it is disassembled into its component small and large ribosomal subunits, deacylated tRNA, and mRNA, allowing these components to enter a new round of translation. Figure adapted with permission from Elsevier, © 2010, from [3].

microscopy was then used to track the position of individual tethered beads as a function of time. From these data, the authors characterized the restricted diffusion of each tethered bead by calculating the root-mean-square horizontal displacement from the average position of the bead (D_{rms}). Addition of phenylalanine-specific tRNA aminoacylated with phenylalanine (Phe-tRNA^{Phe}), the translation elongation factors Tu (EF-Tu) and G (EF-G), and GTP to the adsorbed elongation complexes triggered translation, effectively shortening the length of the mRNA tether and further restricting the diffusion of the beads. By measuring the resulting decrease in D_{rms} as a function of time and modeling the number of translation elongation cycles associated with a specified decrease in D_{rms}, the authors determined that single ribosomes in their experimental system could undergo protein synthesis at a rate of 1–2 peptide bonds s^{-1}, only an order of magnitude slower than the rate of protein synthesis observed *in vivo* [13,14]. The publication of this landmark study marked the beginning of a still-evolving period of rapid progress in single-molecule studies of protein synthesis.

Using a slightly different approach, Puglisi, Funatsu, and co-workers were able to measure the aggregate rate of transcription, translation, co-translational folding, and chromophore maturation (k_{obs}) of green fluorescent protein (GFP) (Figure 2c and d) [15•,16]. To accomplish this, the authors tethered genetically engineered and directly biotinylated ribosomes onto the surface of a quartz microfluidic flowcell. A DNA template encoding a fast-maturing GFP variant followed by an amino acid spacer long enough to extrude GFP through the ribosomal polypeptide exit tunnel and the Secretion Monitor translational arrest sequence was then added to the surface-tethered

Figure 2



Measuring the rate of protein synthesis by single ribosomes. **(a)** The experimental system used by Cooperman, Goldman, and co-workers to measure the rate of polyphenylalanine synthesis by single ribosomes is shown. The 3′-end of the mRNA (black curvy line) from a mica microscope slide-adsorbed ribosomal elongation complex (gray) is labeled with a fluorescently labeled microsphere (pink sphere). The polyphenylalanine peptide is shown in orange. The black dotted line represents the average end-to-end distance of the mRNA tether. The diffusion of the microsphere is restricted to the region bounded by the mica microscope slide and the average end-to-end distance of the mRNA tether, denoted by the green dashed arc and the green shading. Adapted with permission from Cold Spring Harbor Laboratory Press, © 2003, from [11•]. **(b)** Centroid distributions of beads undergoing restricted diffusion. Centroid distributions were measured at 17 min (pink squares), 54 min (orange triangles), and 95 min (black circles) after addition of Phe-tRNA^{Phe}, EF-Tu, EF-G and GTP to the mica-adsorbed ribosomal elongation complexes. Adapted with permission from Cold Spring Harbor Laboratory Press, © 2003, from [11•]. **(c)** The experimental system used by Puglisi, Funatsu, and co-workers to measure the rate of GFP synthesis by single ribosomes is shown. In this system, a ribosome tethered to the flowcell surface translates an mRNA encoding GFP fused to a translational arrest sequence that interacts with the ribosomal exit tunnel, stalls the elongation cycle, and prevents dissociation of the newly synthesized GFP from the ribosome, effectively localizing the GFP to the flowcell surface so that it can be visualized using TIRF microscopy (left panel). A control sample lacking the arrest signal fails to localize the synthesized GFP to the flowcell surface and thus should not yield a detectable signal (right panel). Adapted with permission from Oxford University Press, © 2008, from [15•]. **(d)** TIRF images of surface-localized GFP molecules taken at 5 min (top panels) and 20 min (bottom panels) after delivery of purified translation components to surface-tethered ribosomes programmed with an mRNA encoding GFP fused to the translational arrest sequence (left panels) or encoding GFP lacking the translational arrest sequence (right panels). Adapted with permission from Oxford University Press, © 2008, from [15•].

ribosomes along with a reaction mixture containing the full complement of purified transcription and translation components with the exception of ribosomes. After incubating the reaction for a defined period of time, the flowcell was washed to quench the reaction, and TIRF microscopy was used to image and quantify the number of individual, fully matured GFPs that were anchored to the surface of the flowcell via a translationally stalled ribosome. By varying the incubation time, the authors were able to measure k_{obs}. An analogous solution-based, ensemble reaction using wild-type ribosomes exhibited a similar k_{obs}, suggesting that the genetic engineering and surface tethering of the ribosomes did not significantly alter their biochemical activity. It is important to note, however, that the single-molecule and ensemble reactions were both rate limited by chromophore maturation ($\sim$0.07 min^{-1}), such that small

perturbations to the translational activity of the genetically engineered, surface-tethered ribosomes might not have been detected. Nevertheless, together with a very similar subsequent investigation [17], this study demonstrates the feasibility of using genetically engineered and surface-tethered ribosomes to study translation and co-translational protein folding.

Monitoring multiple rounds of the elongation cycle with single-codon resolution

During the synthesis of a single protein, the ribosome spends the majority of its time and energy on translation elongation, a process that can be divided into three fundamental substeps: aa-tRNA selection [18,19], peptide bond formation [20,21], and translocation [19,22] (Figure 1c). A higher resolution mechanistic understanding of translation elongation than that provided by the experiments described in the previous section can be obtained by observing translation with single-codon resolution and in real time. A major challenge to achieving this using typical TIRF microscopy-based experimental setups (Figure 3a) is the high background fluorescence that arises when fluorescently labeled translation components are delivered into a flowcell containing surface-tethered ribosomes. To maintain acceptably low background fluorescence levels, the concentration of such components is typically limited to <50 nM, concentrations that are two to three orders of magnitude lower than those typically used in *in vitro* ensemble biochemical experiments or found *in vivo*. At such low concentrations, translation becomes rate limited by the low probability of binding of translation components to ribosomes, significantly limiting the mechanistic information that can be accessed by the experiment.

Recently, this challenge has been overcome using two different approaches. The first approach confines the fluorescent labels to the surface-tethered ribosomes and uses a change in a ribosome-based fluorescence signal as a reporter for the transit of the ribosome through individual rounds of the elongation cycle, thus obviating the need to introduce fluorescently labeled translation components into the flowcell [23,24,25••]. To achieve this, a single-molecule fluorescence resonance energy transfer (smFRET) [26,27] signal was developed using ribosomes labeled with a FRET donor fluorophore on the small ribosomal subunit [23] and a FRET acceptor fluorophore on the large ribosomal subunit [24]. At each codon, this smFRET signal cycles between two distinct FRET efficiencies (E_{FRET}); upon peptide bond formation, the smFRET signal transitions from an initial high E_{FRET} to a low E_{FRET}, ultimately reverting to the original high E_{FRET} upon translocation [24]. By monitoring this sequence of high-low-high transitions in E_{FRET} at each codon, Aitken and Puglisi characterized multiple rounds of the elongation cycle with single-codon resolution and in real time [25••].

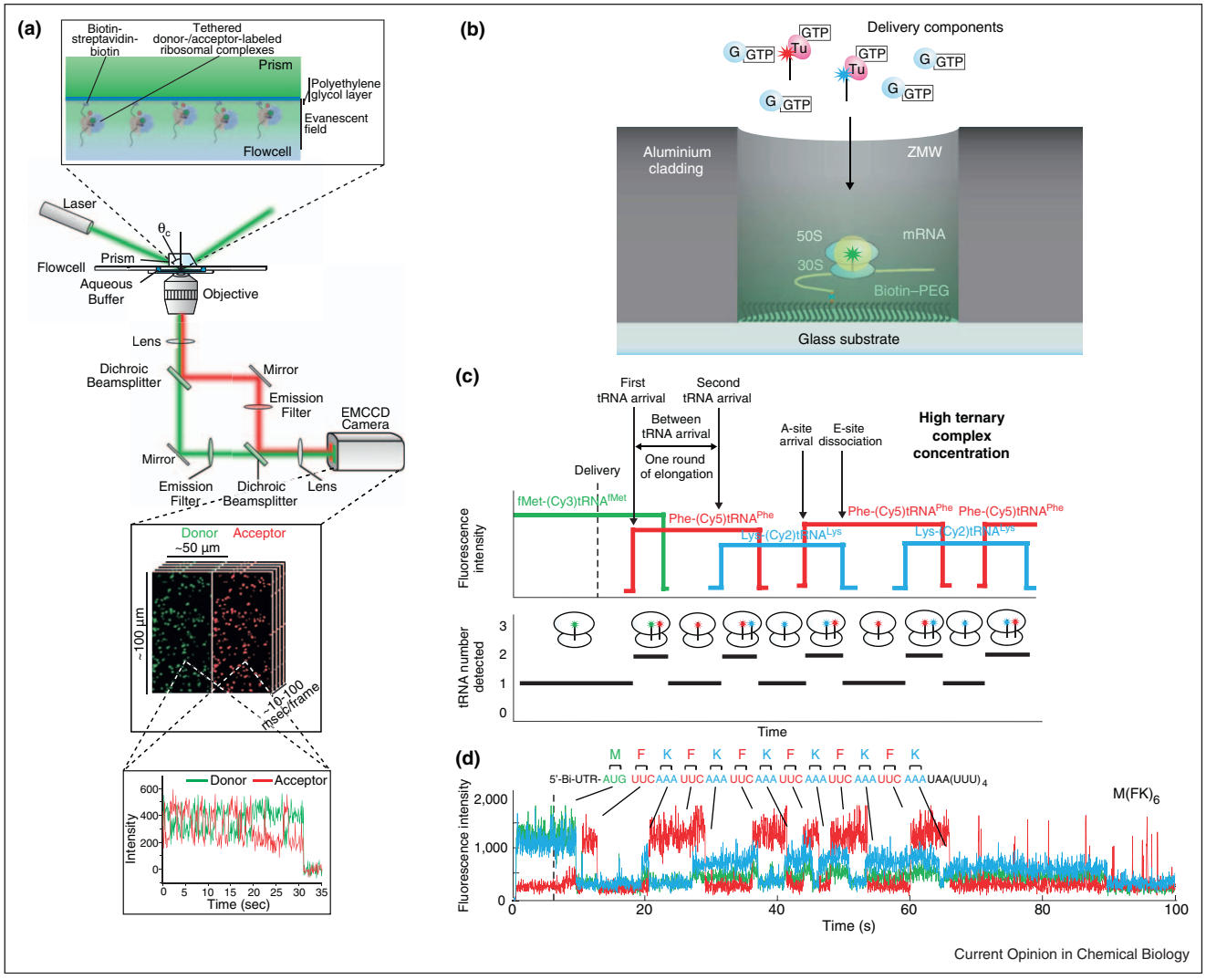
The second approach involves tethering single ribosomes to the bottom of 50–200 nm diameter nanowells, known as zero-mode waveguides (ZMWs) that are nanofabricated into a thin metal film deposited onto the surface of a microfluidic flowcell (Figure 3b–d) [28,29,30••]. Because the diameter of a ZMW is much smaller than the wavelength of light that is used to excite the fluorophores (typically >450 nm), propagation of the excitation light is inhibited, significantly limiting the excitation volume to a few zeptoliters (10^{-21} L) at the bottom of the ZMW, enabling experiments in which fluorescently labeled components can be introduced into the flowcell at physiological or near-physiological concentrations [28,29]. Using this approach, Puglisi and coworkers were able to work with fluorescently labeled fMet-tRNA^{fMet}, Phe-tRNA^{Phe}, and Lys-tRNA^{Lys} at concentrations of up to 500 nM each, allowing them to observe the real-time transit of multiple tRNAs through single, actively translating ribosomes with single-codon resolution [30••].

Collectively, the two experimental approaches described above have provided mechanistic details into the molecular basis for the processivity of the ribosome [25••], the origins of global translational effects induced by ribosome-targeting antibiotics [25••], and the coupling between tRNA binding and dissociation events on single, actively translating ribosomes [30••]. Looking forward, these experimental approaches hold particular promise for investigations of recoding, a set of critical, but mechanistically poorly defined, regulatory events in which ribosomes undergo a +1 or −1 frameshift, miscode a sense codon, or read through a stop codon at a precise location within an mRNA [31].

Kinetic dissection of partial reactions within the translation process

Even higher resolution mechanistic information can be accessed by observing individual ribosomes as they undergo partial reactions, such as aa-tRNA selection and translocation, within a single elongation cycle (Figure 1c). Using ribosomal elongation complexes carrying either a donor-labeled peptidyl-tRNA [32••,33–38] or a donor-labeled ribosome [37], several groups have directly observed acceptor-labeled aa-tRNA selection in real time and have probed the response of individual ribosomes to the delivery of aa-tRNAs: firstly, to mRNA codons containing one or more base pair mismatches relative to the aa-tRNA anticodon (known as near-cognate and non-cognate codons, respectively) [32••,33,37,38]; secondly, in the presence of ribosome-targeting antibiotics that perturb aa-tRNA selection [32••,33,34,37]; and/or thirdly, that are misacylated with incorrect amino acids [35]. Collectively, these smFRET experiments have revealed new reaction intermediates, transiently sampled conformational states, and thermally activated structural fluctuations that are important aspects of the mechanism of aa-tRNA selection,

Figure 3



Total internal reflection fluorescence (TIRF) microscopy and zero-mode waveguides (ZMWs). **(a)** A typical TIRF system for smFRET studies of translation is shown. A laser excitation source is totally internally reflected at the interface formed between the quartz microfluidic flowcell and the aqueous buffer in which ribosomal complexes are tethered. This results in the generation of an evanescent field that propagates into the buffer and decays exponentially as a function of increasing distance from the quartz–buffer interface, thereby selectively exciting FRET donor fluorophores on ribosomal complexes that are localized within ~300 nm of the quartz–buffer interface (top inset). Fluorescence emission from the FRET donor and acceptor fluorophores is collected by an objective, wavelength-separated using dichroic beamsplitters, and directed at an electron-multiplying charge-coupled device (EMCCD) camera for detection (middle inset). The separated donor and acceptor fluorescence intensities emerging from single, optically resolved ribosomal complexes can then be quantified and plotted as a function of time (bottom inset). **(b)** The experimental system used by Puglisi and co-workers to monitor multiple rounds of the elongation cycle with single-codon resolution using ZMWs is shown. A ribosomal initiation complex carrying a P site-bound, Cy2-labeled fMet-tRNA^{fMet} and programmed with an mRNA encoding fMet (M) followed by a series of Phe (F)-Lys (K) repeats is tethered to the bottom of the ZMW, and translation is triggered by the delivery of Cy5-labeled Phe-tRNA^{Phe}, Cy2-labeled Lys-tRNA^{Lys}, EF-Tu, EF-G, and GTP. Three laser lines with wavelengths of 488 nm, 532 nm, and 642 nm were simultaneously used to directly excite the Cy3, Cy5, and Cy2 fluorophores, respectively, and the fluorescence emission from all three fluorophores was simultaneously detected. Adapted with permission from Nature Publishing Group, © 2010, from [30••]. **(c)** Schematic of the results expected from the experiment described in (b). The top panel depicts a plot of the expected Cy3 (green), Cy5 (red), and Cy2 (blue) fluorescence emission pulses versus time trajectory that is generated as Cy5-labeled Phe-tRNA^{Phe} and Cy2-labeled Lys-tRNA^{Lys} are alternately delivered and transit through a ribosomal complex initially carrying a P site-bound, Cy3-labeled fMet-tRNA^{fMet}. The first few tRNA arrival and dissociation events as well as the first round of the elongation cycle are denoted by arrows. The bottom panel plots the transit of tRNAs through the ribosomal complex as a function of time as determined by an analysis of the plot of the expected Cy3, Cy5, and Cy2 fluorescence emissions versus time shown in the top panel. Adapted with permission from Nature Publishing Group, © 2010, from [30••]. **(d)** A representative, experimentally observed Cy3, Cy5, and Cy2 fluorescence emission pulses versus time trajectory obtained from performing the experiment was described in (b) and schematized in (c). This particular trajectory was recorded using 200 nM concentrations of both Cy5-labeled Phe-tRNA^{Phe} and Cy2-labeled Lys-tRNA^{Lys}. Adapted with permission from Nature Publishing Group, © 2010, from [30••].

but that have been difficult or impossible to characterize using ensemble biochemical approaches or structural studies.

Similarly, the mechanism of translocation has been investigated by several groups employing smFRET signals developed using fluorescently labeled ribosomes, tRNAs, and/or EF-G [24,39••,40–42,43••,44••,45•,46•,47–55]. The majority of these studies have revealed that following peptide bond formation, the ribosome and ribosome-bound tRNAs undergo large-scale, thermally activated structural fluctuations that allow the entire ribosomal pre-translocation complex to transiently sample a critical structural intermediate on the translocation pathway [39••,40–42,43••,44••,45•,46•,47–55]. Detailed studies of the effect that Mg^{2+} concentration [42], temperature [53], ribosome–tRNA interactions [43••,44••,45•,46•,55], tRNA structure and stability [55], small-molecule translocation inhibitors [24,41,42,44••,48–52], and EF-G binding [41,43••,44••,45•,46•,49,50,52,54,55] have on these dynamics strongly suggest that translocation is at least partially driven by a Brownian motor mechanism. In this view, the architecture of the ribosome, the structure and stability of the ribosome-bound tRNAs, and the nature of ribosome–tRNA interactions collaborate so as to bias the thermally activated fluctuations of the pre-translocation complex towards formation of the relevant translocation intermediate. Binding of EF-G to the pre-translocation complex then transiently stabilizes this intermediate as part of the mechanism through which EF-G promotes the translocation reaction.

Expanding beyond the elongation cycle, smFRET studies are also providing significant insight into the mechanisms of partial reactions within the initiation [56], termination [57], and ribosome recycling [57] stages of protein synthesis (Figure 1b). Taken together with the growing body of ensemble biochemical studies of these partial reactions and structural investigations of functional ribosomal complexes involved in these stages of protein synthesis (reviewed recently in [18,58–65]), we expect that ongoing and future smFRET investigations will help better define the mechanisms that govern each of the stages of protein synthesis.

In vivo imaging of ribosome-catalyzed protein synthesis

Despite the enormous potential that fluorescence imaging holds for enabling mechanistic studies of complex biochemical reactions in their native environments within living cells, the application of *in vivo* fluorescence imaging techniques to single-molecule studies of protein synthesis has progressed at a much slower rate than their *in vitro* counterparts described above. This is primarily because of technical challenges, including cellular autofluorescence [66], the limited brightness of genetically encodable fluorescent proteins [66], the relatively small number of methods that allow more photophysically robust organic fluorophores to be genetically encoded [67], and the dearth of cell membrane-permeable organic fluorophores suitable for single-molecule imaging [66], among others that must be overcome in order to localize single fluorophores with high spatial and temporal resolution in living cells. Nevertheless, a handful of studies over the past several years have successfully overcome these challenges, introducing methods for *in vivo* single-molecule fluorescence imaging of ribosomes and protein synthesis.

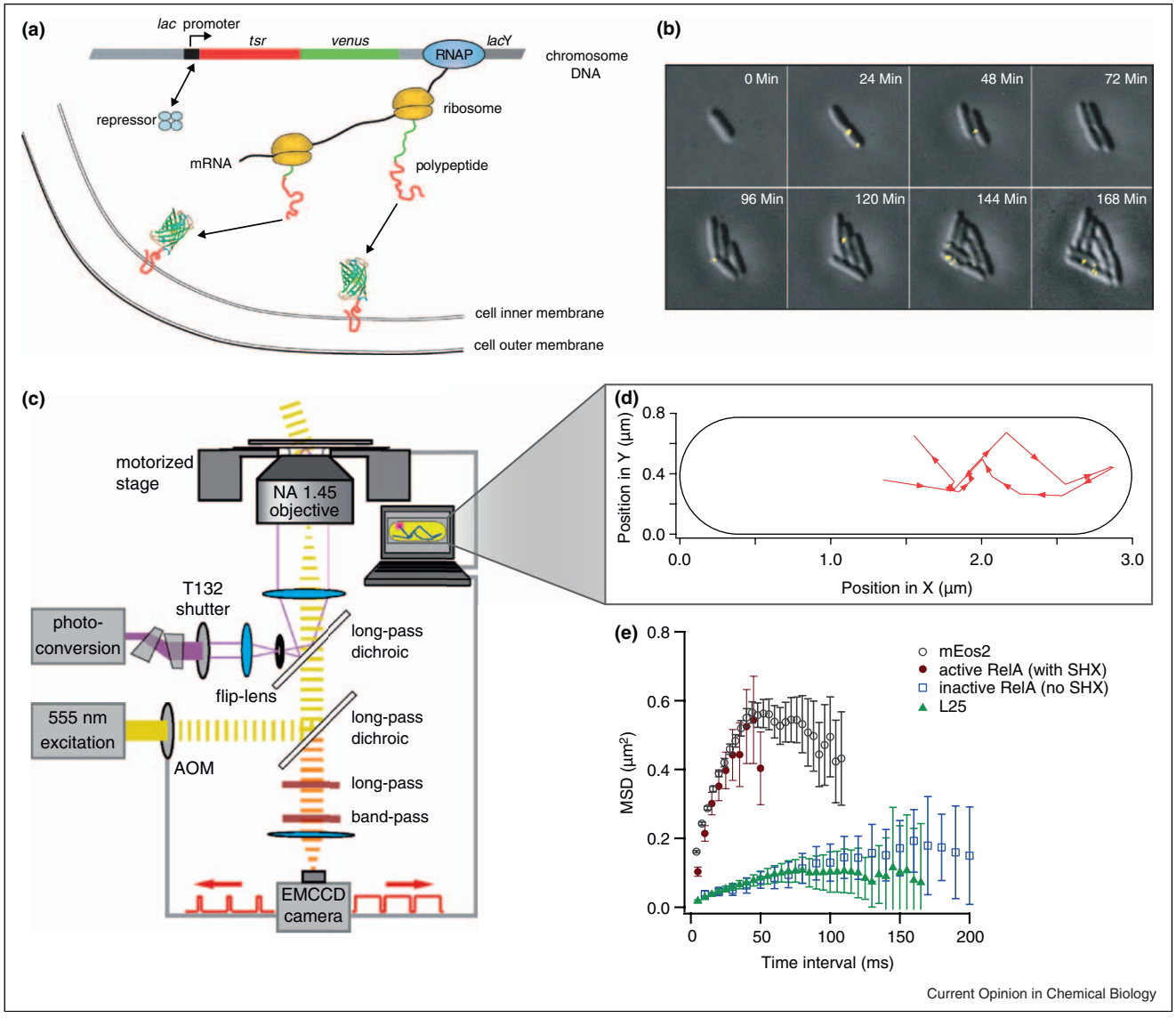
Quantifying gene expression in living cells

In a series of groundbreaking articles over the past five years, Xie and co-workers have successfully imaged the synthesis of single protein molecules *in vivo* [68,69•,70]. In the first of these articles, the authors quantified the number and timing of single protein synthesis events in live *Escherichia coli* cells (Figure 4a and b) [69•]. This was accomplished using a cell line in which the native lacZ gene that is naturally under the control of a repressed *lac* promoter was replaced with a single copy of a gene encoding the membrane-targeting protein Tsr fused to a fast-maturing variant of yellow fluorescent protein (YFP). By monitoring the appearance of fluorescence from individual, membrane-bound Tsr-YFP proteins arising from the translation of single mRNA molecules that were themselves produced by the rare and spontaneous dissociation of *lac* repressor from the operator region of the *tsr-yfp* gene, the authors demonstrated that protein synthesis under these conditions proceeds through randomly occurring and temporally uncorrelated bursts of protein production. For this particular genetic construct, individual cells exhibited an average of 1.2 bursts per cell cycle with an average of 4.2 Tsr-YFP molecules produced per burst. The relatively long waiting times between the observation of single Tsr-YFP proteins within a burst (originating from the characteristically slow chromophore maturation time of YFP) and the considerably slow diffusion of individual, membrane-bound Tsr-YFP proteins allowed this study to be performed using relatively conventional epifluorescence microscopy with diffraction limited spatial resolution and long image acquisition times. Together with a number of follow up studies [68,70], Xie and co-workers have demonstrated that random events, which can only be characterized using single-molecule approaches, play decisive roles in driving gene expression, regulating the composition of the proteome, and controlling the observed phenotype of living cells.

High-resolution tracking of ribosomes and ribosome-associated factors in living cells

Expanding upon the groundwork laid by Xie and co-workers, Dekker, Elf, and co-workers have used photoconvertible GFPs (pcGFPs) and a stroboscopic time lapse imaging approach to track ribosomes and a ribosome-associated factor known as RelA with a sub-diffraction

Figure 4



In vivo imaging of ribosomes and protein synthesis. **(a)** The experimental system used by Xie and co-workers to quantify gene expression in living cells is shown. Rare and spontaneous dissociation of *lac* repressor from the operator region of the *tsr-yfp* gene allows for RNA polymerase binding and transcription, yielding a single mRNA transcript. Translation of the mRNA transcript, folding and membrane insertion of the resulting Tsr-YFP protein, and maturation of the YFP chromophore allows single, membrane bound Tsr-YFP proteins to be detected by fluorescence microscopy. Adapted with permission from The American Association for the Advancement of Science, © 2006, from [69•]. **(b)** A sequence of images obtained using epifluorescence microscopy (yellow) overlaid with images obtained from differential interference contrast (DIC) optical microscopy (grayscale) of *E. coli* cells expressing Tsr-YFP as described in (a). The eight images shown here were obtained from a single field-of-view and were taken over 168 min using a 100 ms exposure time for each image. After each image was recorded, an 1100 ms laser pulse was applied to photobleach the fluorophores in preparation for the next round of imaging. Adapted with permission from The American Association for the Advancement of Science, © 2006, from [69•]. **(c)** The experimental system used by Dekker, Elf, and co-workers to track ribosomes and ribosome-associated protein factors in living cells is shown. A short pulse from a violet photoconversion laser is periodically used to stochastically photoconvert one or a few photoconvertible GFPs (pcGFPs). Photoconverted pcGFPs are imaged using an acousto-optical modulator (AOM) to generate short pulses of a yellow excitation laser that are synchronized with an EMCCD camera such that the laser pulses occur in the middle of each imaging frame. Adapted with permission from The National Academy of Sciences, © 2011, from [71••]. **(d)** A representative path obtained by tracking a freely diffusing cytosolic pcGFP. The analysis of such paths allows the mean square displacement (MSD) to be calculated and, ultimately, a diffusion coefficient to be determined. Adapted with permission from The National Academy of Sciences, (c) 2011, from [71••]. **(e)** A plot of the MSDs as a function of the time interval between images is shown. Of the four data sets, two, pcGFP (mEos2) and pcGFP-labeled ribosomes (L25), provide controls representing a freely diffusing cytosolic protein and a subdiffusing ribosome, respectively. Under amino acid-rich growth conditions, the MSD as a function of the time interval obtained for RelA-pcGFP corresponds well with that observed for pcGFP-labeled ribosomes, demonstrating that under amino acid-rich conditions, RelA remains bound to ribosomes. However, under L-serine hydroxamate (SHX)-induced starvation conditions, the MSD as a function of the time interval obtained for RelA–pcGFP correlates more closely with that observed for cytosolic pcGFP. This latter result suggests that, under the stress induced by amino acid starvation, RelA dissociates from the ribosome and, while free in the cytosol, synthesizes the ppGpp alarmone associated with the stringent response. Adapted with permission from The National Academy of Sciences, © 2011, from [71••].

spatial resolution of ~44 nm and a time resolution of 4–50 ms (Figure 4c–e) [71••]. To accomplish this, the authors generated three *E. coli* cell strains expressing free pcGFP, ribosomes carrying a ribosomal protein L25–pcGFP fusion protein, or a RelA–pcGFP fusion protein, respectively. By stochastically photoconverting only one or a few pcGFPs at a time and synchronizing short laser excitation pulses with the frame time of their camera, individual photoconverted pcGFPs are rendered optically immobile within each imaging frame and can be localized with subdiffraction-limit resolution. Using this approach, the authors demonstrated that free pcGFPs exhibit free diffusion characterized by a microscopic diffusion coefficient of ~13 $\mu m^2 s^{-1}$ whereas pcGFP-labeled ribosomes exhibit subdiffusive behavior characterized by a microscopic diffusion coefficient of ~0.5 $\mu m^2 s^{-1}$. In addition, the data suggest that the observed subdiffusive behavior of the ribosomes results from tethering to mRNAs that are in turn tethered to DNA during coupled transcription–translation in *E. coli*.

Using the RelA–pcGFP protein construct, the authors were able to demonstrate that RelA, a factor involved in the synthesis of the ppGpp alarmone during the starvation-induced stringent response in *E. coli*, is tightly bound to ribosomes during amino acid rich conditions and dissociates from ribosomes for prolonged periods of time (i.e. hundreds of ms) under conditions of amino acid starvation. These observations led the authors to conclude that synthesis of ppGpp by RelA occurs off, rather than on, the ribosome and does not require repetitive binding and dissociation of RelA from ribosomes, thereby resolving long-standing questions regarding the mechanism of RelA function during the stringent response [72]. Given the abundance of aa-tRNAs, translation factors, and regulatory factors that interact with the ribosome during protein synthesis, it is quite easy to envision how stroboscopic single-molecule tracking of ribosome-associated factors beyond RelA would provide a greater mechanistic understanding of translation *in vivo*. For example, applications of this approach should make it possible to characterize the mechanism and regulation of translation initiation (Figure 1b), the organization and timing of aa-tRNA binding and dissociation from elongating ribosomes, and the mechanism of coupled transcription–translation, among others.

Conclusions

While current *in vitro* and *in vivo* single-molecule fluorescence imaging studies have greatly contributed to our mechanistic understanding of ribosome-catalyzed protein synthesis, much remains to be learned about the mechanism and regulation of translation. As briefly mentioned in the preceding sections, we expect that these techniques will continue to expand into studies of co-translational protein folding; regulatory events such as recoding; and the initiation, termination, and ribosome recycling stages of protein synthesis. While, the single-molecule fluorescence imaging approaches described in this article have thus far remained confined to *E. coli*-based translation systems and *E. coli* cells, recent reports describing an *in vitro* reconstituted yeast translation system [73], an X-ray crystal structure of the yeast ribosome [74], and a strategy for fluorescently labeling yeast ribosomes [75] should facilitate single-molecule fluorescence imaging of eukaryotic ribosomes and protein synthesis. These approaches will be especially valuable for elucidating the timing and organization of molecular events during eukaryotic translation initiation, a highly dynamic process in which approximately thirteen eukaryotic initiation factors direct the assembly of a ribosomal initiation complex that is primed to enter the elongation cycle [76].

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- • of special interest
- •• of outstanding interest

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