Elucidating mechanistic principles underpinning eukaryotic translation initiation using quantitative fluorescence methods

Abigail L. Stevenson, Pedro P. Juanes[1] and John E.G. McCarthy[1]
Manchester Interdisciplinary Biocentre, University of Manchester, 131 Princes Street, Manchester M1 7DN, U.K.

Abstract

Eukaryotic translation initiation is an intricate process involving at least 11 formally classified eIFs (eukaryotic initiation factors), which, together with the ribosome, comprise one of the largest molecular machines in the cell. Studying such huge macromolecular complexes presents many challenges which cannot readily be overcome by traditional molecular and structural methods. Increasingly, novel quantitative techniques are being used to further dissect such complex assembly pathways. One area of methodology involves the labelling of ribosomal subunits and/or eIFs with fluorophores and the use of techniques such as FRET (Förster resonance energy transfer) and FA (fluorescence anisotropy). The applicability of such techniques in such a complex system has been greatly enhanced by recent methodological developments. In the present mini-review, we introduce these quantitative fluorescence methods and discuss the impact they are beginning to have on the field.

Eukaryotic translation initiation: a complex macromolecular assembly pathway

The synthesis of all cellular proteins is performed by ribosomes, in a process called translation. The key steps of the translation cycle are conserved in all domains of life: initiation, elongation, termination and ribosome recycling. In the present mini-review, we focus on the fundamental process of initiating translation of a protein molecule from the start codon of an mRNA in the active site of a ribosome. In bacteria, translation initiation is aided by only three factors [IFs (initiation factors) 1–3)] and a formylated initiator tRNA (fMet-tRNA$_i^{fMet}$), which collectively facilitate start codon recognition and the transition into the peptide elongation cycle. As illustrated in Figure 1, the situation in a eukaryotic organism such as yeast is more complex, with a larger (80S) ribosome, a capped and polyadenylated mRNA lacking an equivalent to the bacterial SD (Shine–Dalgarno) sequence and more than 11 eIFs (eukaryotic IFs). In higher eukaryotes, even more factors have evolved in parallel with the increased complexity of gene expression and regulation in mammalian and plant cells. For example, a number of ITAFs [IRES (internal ribosome entry site) *trans*-acting factors] have been identified that promote initiation at IRESs ([1] and reviewed in [2]).

Key words: biophysics, eukaryotic ribosome, Förster resonance energy transfer (FRET), translation initiation.

Abbreviations used: AFP, autofluorescent protein; eIF, eukaryotic initiation factor; EM, electron microscopy; FA, fluorescence anisotropy; FRET, Förster resonance energy transfer; GFP, green fluorescent protein; IF, initiation factor; IRES, internal ribosome entry site; MFC, multifactor complex; TC, ternary complex; TCM, tetracysteine motif.

[1]**To whom correspondence should be addressed at the present address:** School of Life Sciences, University of Warwick, Coventry CV4 7AL, U.K. **(email john.mccarthy@warwick.ac.uk).**

Research over the last 20 years has determined that these eIFs bind in a dynamic stepwise manner to position the mRNA's start codon in the peptidyl (P) site of the 80S ribosome to initiate translation (Figure 1; for more detail, see [3]). Briefly, an initiator tRNA aminoacylated with methionine (Met-tRNA$_i^{Met}$) binds to heterotrimeric eIF2–GTP to form the TC (ternary complex). The TC then combines with eIF1, the large multisubunit factor eIF3 and possibly eIF5 to form the MFC (multifactor complex), a structure that already exceeds 1 MDa [4]. Through interactions with eIF3 and the addition of eIF1a, the MFC now joins with the small 40S subunit of the ribosome to form the 43S complex. Recruitment of capped mRNA is mediated by the cap-binding complex (eIF4F), whereupon the 40S subunit scans along the mRNA in a 5′→3′ direction until a start codon is reached; then, eIF5B aids the joining of the large 60S ribosomal subunit, resulting in eIF5-mediated GTP hydrolysis and factor release, allowing the 80S ribosome to begin the elongation phase of translation [5,6].

This model of eukaryotic translation initiation has been formulated on the basis of studies from various groups using a combination of traditional methods (yeast genetics, *in vitro* reconstitution translation assays, and chemical and enzymatic footprinting) plus a range of biophysical methods [including ITC (isothermal calorimetry), crystallography, NMR, SPR (surface plasmon resonance) and AFM (atomic force microscopy)]. However, only the basics are known about the eukaryotic pathway, leaving many important details of eIF binding and ribosome subunit joining, as well as the conformational dynamics of the eukaryotic ribosome, to be elucidated.

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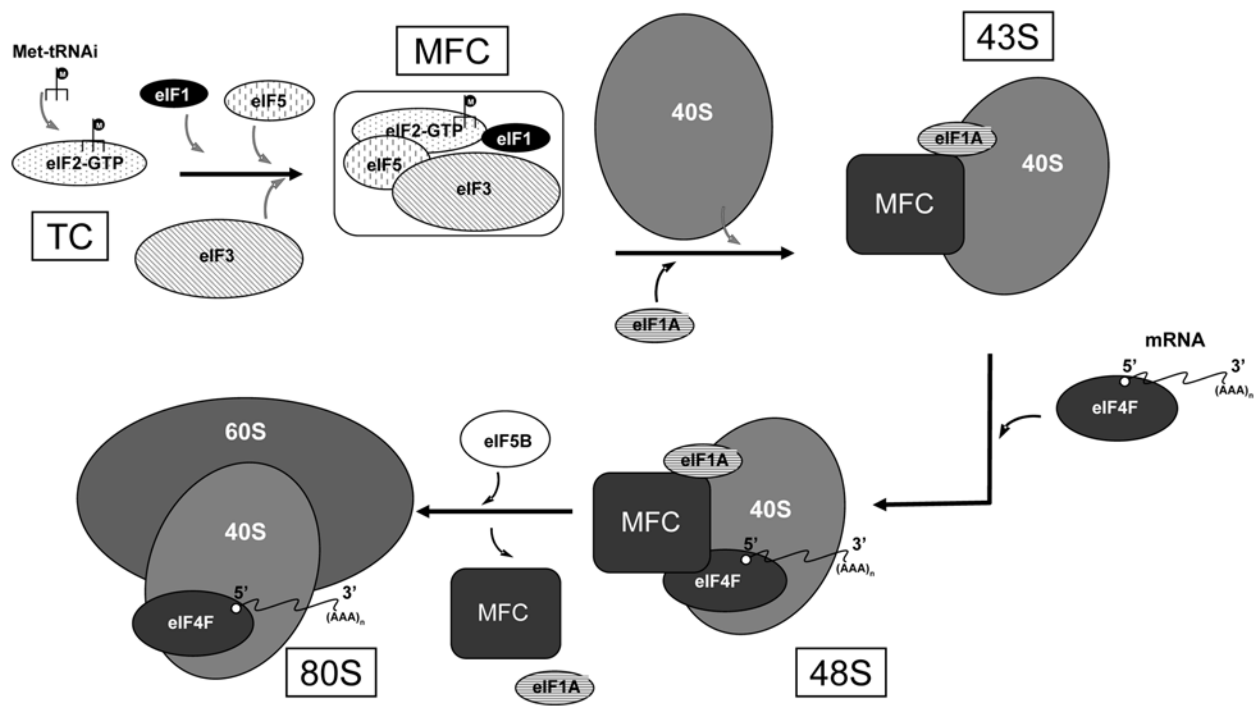
Figure 1 | Simplified schematic diagram of eukaryotic translation initiation in yeast

An initiator tRNA aminoacylated with methionine (Met-tRNA$_i^{Met}$) binds to eIF2–GTP to form the TC. The TC then combines with eIF1, the large multisubunit factor eIF3 and possibly eIF5 to form the MFC. Through interactions with eIF3 and the addition of eIF1A, the MFC now joins with the small 40S subunit of the ribosome to form the 43S complex. mRNA that is capped at the 5′-end (white circle) and polyadenylated at the 3′-end [(AAA)$_n$] may be circularized by the binding of the eIF4F complex to the cap and poly(A)-binding protein (PABP) to the poly(A) tail (for clarity, PABP and circularization is not shown). The mRNA binds to the 43S complex via eIF4F to form the 48S complex. Now, the 40S subunit can scan along the mRNA in a 5′→3′ direction until a start codon is reached; eIF5B then aids the joining of the large 60S ribosomal subunit, resulting in GTP hydrolysis, factor release and allows the 80S ribosome to begin the elongation phase of translation.



The eukaryotic ribosome: a highly challenging target for structural biology

The basic two-subunit structure of the ribosome is highly conserved in bacteria and eukaryotes, but the eukaryotic 80S ribosome is a larger 4.2 MDa complex comprising a 60S subunit (three ribosomal RNAs and ∼49 ribosomal proteins) and a 40S subunit (one ribosomal RNA and ∼33 ribosomal proteins). The manufacture of such a huge ribonucleoprotein machine requires a complicated cellular assembly pathway, which itself involves numerous chaperone-like RNA–protein complexes {snoRNPs (small nucleolar ribonucleoprotein particles), reviewed in [7]}.

The more compact bacterial 70S ribosome has been crystallized; first as vacant individual subunits published in 2000 [8–10], and then in 2006 in combination with all three tRNAs and mRNA, to 2.8 Å (1 Å = 0.1 nm) resolution [11]. These studies allow us to visualize the ribosome at various stages of the initiation pathway in atomic detail (Ramakrishnan, Steitz, Noller and Yonath groups; for a comprehensive review, see [12]). Such groundbreaking crystal structures and the resulting molecular insights into prokaryotic translation earned Tom Steitz, Ada Yonath and Venki Ramakrishnan the Nobel Prize in Chemistry in 2009.

Sadly, such detailed atomic information on the eukaryotic ribosome is lacking, as no crystal structure of the 80S ribosome or any of its constituent parts has so far been obtained. This may partly be due to the increased complexity and flexibility of the 80S ribosome, partially attributable to the eukaryotic rRNA-specific ‘expansion segments’. Another major challenge here is that the addition of the numerous eIFs required for 40S–mRNA binding induces significant conformational changes, and thus heterogeneity within the ribosomal subunit population, and this may prevent crystallization [13]. Single-particle cryo-EM (cryo-electron microscopy) is an alternative structural technique that can provide lower-resolution structural information on large dynamic macromolecular structures, such as the eukaryotic ribosome. However, where there is such conformational heterogeneity, high-resolution reconstructions (lower than 10 Å) are difficult to achieve. 80S reconstructions have been successful to 7.3 Å [14]. However, complexes of 40S with IFs seem to show a comparatively high degree of conformational flexibility, but so far only low-resolution

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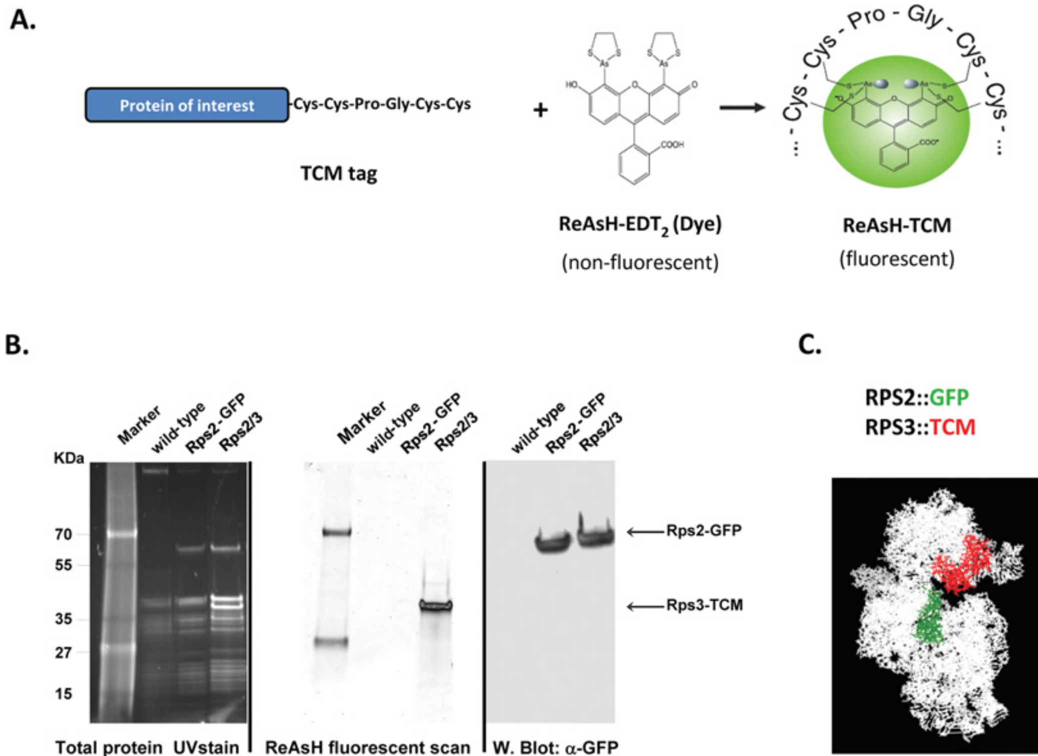
Figure 2 | Single and double labelling of the 40S subunit using GFP and TCM tags

(**A**) TCM tags bind biarsenical dyes, such as ReAsH-EDT$_2$. (**B**) Total protein UV staining, ReAsH labelling and Western blotting confirm the presence of both tags in Rps2/3 ribosome preparations labelled with ReAsH. Molecular masses are indicated in kDa. (**C**) Position of tagged Rps proteins highlighted on a cryo-EM map of the 40S ribosomal subunit, created using Chimera software (UCSF).



maps have been attained [13,15]. Although these cryo-EM studies provide valuable information on intra-ribosome conformational changes, other biophysical methods are needed to confirm and dissect these conformational changes in greater molecular detail.

A complementary approach: quantitative fluorescence methods

In this section, we summarize briefly the use of quantitative fluorescence techniques as a strategy to help to elucidate the details of intermolecular interactions and intramolecular conformational changes involved in translation.

Fluorescent labelling of eIFs and ribosomes

Labelling essential cellular proteins with fluorophores without compromising their function is a major challenge for biophysical studies of the translation machinery. Site-directed labelling of cysteine residues with fluorescent dyes using maleimide chemistry is feasible with smaller proteins (eIF1, eIF1A, eIF5B [5,16]), but this is more difficult with larger factors and a different approach has been required. Genetically encoded tags are easy to incorporate; however, the commonly used AFPs (autofluorescent proteins) represent relatively large (~27 kDa), potentially function-altering, additions; indeed, we have found that many eIFs are no longer functional when tagged with such a moiety. Likewise, few ribosomal proteins can be successfully GFP (green fluorescent protein)-tagged in yeast while maintaining viability (Rps2, Rps3, Rpl5 and Rpl25 [17]; Rpl7a/7b and Rps18a/18b [18]). The SNAP^{TM} and CLIP^{TM} tags developed by Jonsson and colleagues [19] allow a plethora of different fluors to be covalently attached to the protein of interest; however, at 24 kDa, they are still bulky additions. An alternative smaller genetically encoded tag incorporates the TCM (tetracysteine motif), which comprises only 13 amino acids and can covalently bind the biarsenical dyes, ReAsH and FlAsH [20] (Figure 2A). Small tags such as the TCM tag are attractive for use in labelling essential proteins such as those of the translation machinery and we have been exploring their use quite extensively.

The complete *in vitro* reconstitution of functional eukaryotic ribosomes from constituent rRNAs and labelled ribosomal proteins has yet to be achieved. We have therefore made use of yeast genetics to create strains from which suitably modified ribosomes and translation factors can be isolated. We have successfully combined AFP and

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TCM tagging to obtain viable yeast strains expressing 40S ribosomes double-labelled with GFP and ReAsH (P.P. Juanes and A.L. Stevenson, unpublished work) (Figure 2). Recently, an alternative approach to labelling eukaryotic ribosomes using fluorescent oligonucleotides hybridized to external loops in rRNA has been developed [21].

Fluorescence methods for studying translation initiation complexes

Suitably labelled components can be used to study eIF–ribosome binding kinetics and conformational dynamics using quantitative fluorescence methods, such as FRET (Förster resonance energy transfer) and FA (fluorescence anisotropy). The interaction of eIF1 and eIF1A within the 43S complex has been studied successfully using FRET, where donor and acceptor fluorophores were placed on two interacting proteins (eIF1 and eIF1A [16]). As illustrated in Figure 3(A), when the donor is in an excited state and the two fluors are within the Förster distance of each other (which depends on the labels used, but typically ∼100 Å), FRET occurs through non-radiative dipole–dipole coupling (for a review of FRET and its applications, see [22]). The resulting increase in acceptor fluorescence and corresponding decrease in donor fluorescence can be detected in a steady-state FRET experiment using standard fluorescence spectrophotometry. Since FRET depends on the molecular distance between the two fluors, a change in FRET efficiency between labelled eIFs or ribosomal proteins can be used to demonstrate protein interactions or intramolecular conformational changes. For example, the steady-state FRET efficiency between labelled eIF1 and eIF1A changed significantly upon binding of both TC and mRNA to a 40S–eIF1–eIF1A complex, suggesting that a conformational change occurs during formation of the eukaryotic translation pre-initiation complex [16]. More accurate measurements of molecular distances in Å can be achieved by determining the change in the donor/acceptor fluorescence lifetime using a laser-equipped fluorimeter. Lifetime measurements, as opposed to total intensity measurements, have the advantage of being concentration-independent; FRET can be measured as it leads to a shortening of the donor lifetime.

Another quantitative fluorescence technique useful for studying large complexes is FA. FA measures the rotational (tumbling) time of a labelled species in solution, determined using a polarizing spectrophotometer (Figure 3C) (for a detailed review, see [23]). Measuring the change in steady-state anisotropy when a labelled factor binds can be used to study interactions of the translation machinery; steady-state FA experiments were used by Doudna and colleagues to study the binding of smaller eIF3j to the larger 40S ribosome [24]. In addition, by measuring the anisotropy lifetime decay time using laser-excited flourimetry, the rotational time of a labelled protein can be determined accurately, gaining more quantitative data on binding kinetics. However, developing FA for use with the larger eIFs is complicated by the fact that many of these proteins have long tumbling times in solution. Thus conventional fluors such as Cy3 (indocarbocyanine) or GFP are unsuitable as labels because they have sub-10 ns fluorescence lifetimes. We have accordingly begun using the recently developed SNAP/CLIP tagging technique [19], which allows the user to incorporate fluors with longer lifetimes. For example, we have been able to label the eIF3 complex with PURETIME®325 (Assaymetrics), which has a fluorescence lifetime of 325 ns. Using this fluor, we have used FA to show that the core eIF3 complex binds to the 40S subunit *in vitro* and have measured the kinetics of this process (Figure 3D) (P.P. Juanes and A.L. Stevenson, unpublished work). However, the introduction of further novel fluors with lifetimes in the microsecond range would be most advantageous for this and other types of biomolecular interaction study.

Both FRET and FA have been applied successfully in stopped-flow experiments to monitor binding kinetics in the prokaryotic translation machinery (for a detailed review on stopped-flow methodology, see [25]). Attaching a stopped-flow instrument to a fluorimeter allows the rapid (less than 1 ms) mixing of two components in solution before beginning observations; these time-resolved experiments allow the determination of accurate reaction rate constants *in vitro*. For example, the guanine-nucleotide-binding properties of IF2 were determined by fluorescence stopped-flow using fluorescent derivatives of GTP/GDP [26]. Once the hurdles associated with labelling the larger components of the eukaryotic translation machinery have been overcome, these fast reaction methods will be very useful to generate a large body of important quantitative data for this more complex system (for example, association and dissociation constants), thus filling in many gaps in our knowledge of the translation pathway.

Fluorescence techniques for studying conformational changes in the ribosome

The labelling strategy that we have developed can also be applied to studying conformational changes within large macromolecular complexes such as the ribosome. This has already been achieved in the prokaryotic system, where ribosomal proteins were labelled in both the 30S and 50S ribosomal subunits [27]. This FRET study revealed the inter-subunit rotation of the large and small subunits during translation elongation. Using the double-labelled 40S ribosomes described previously (Figure 2B), we have performed a similar experiment using FRET to demonstrate the rotation of the 40S head upon MFC binding (Figure 3B) (P.P. Juanes and A.L. Stevenson, unpublished work). These data are consistent with our previous cryo-EM study that suggested a significant rearrangement of the 40S structure upon formation of the 43S complex [13].

Conclusions and future developments

Quantitative fluorescence techniques complement structural analysis to provide a deeper understanding of the complex and dynamic interactions involved in the translation initiation

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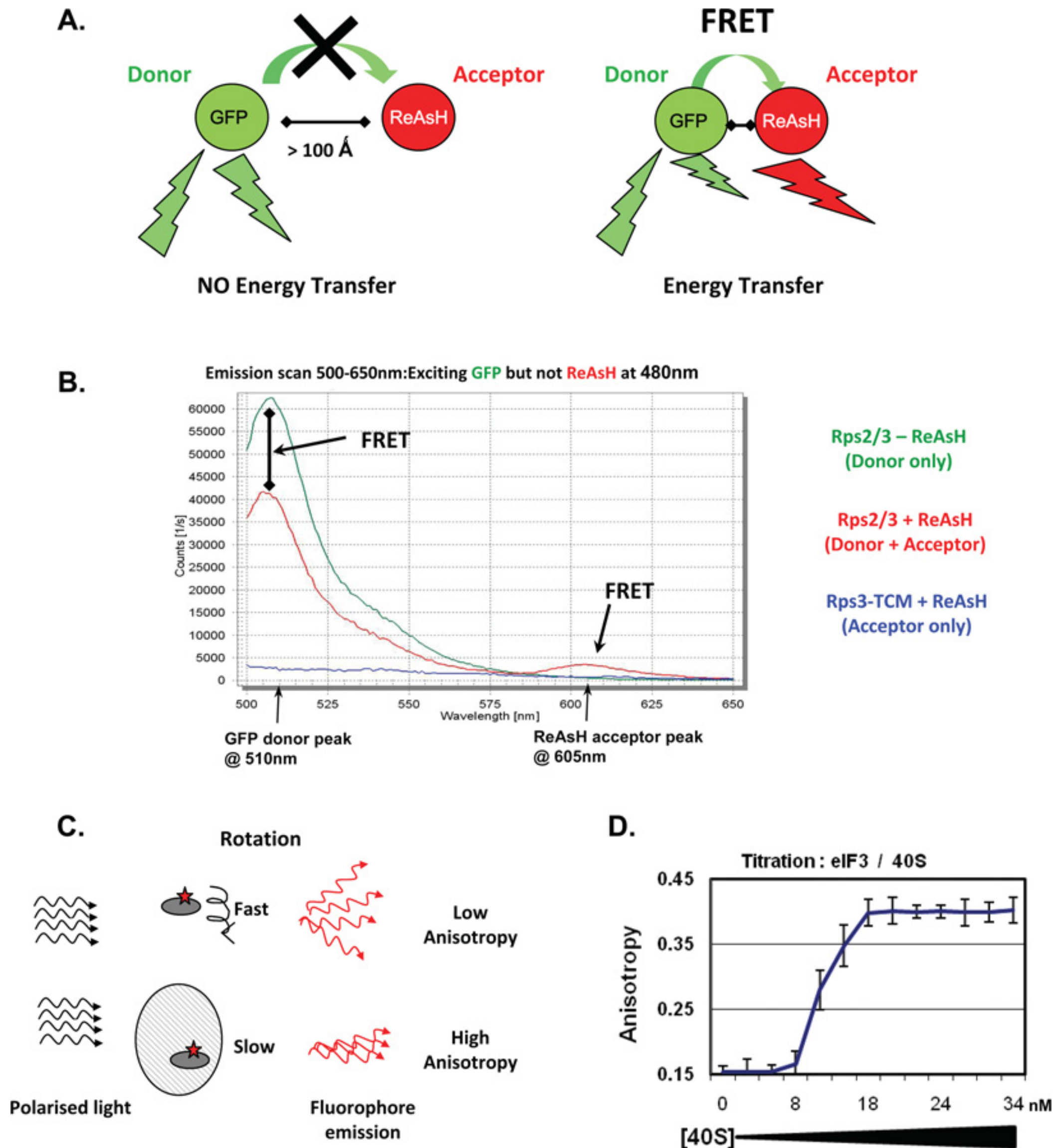
Figure 3 | Fluorescence methods for studying the translation machinery

(**A**) Basic principle of FRET: when two fluorophores with overlapping spectra come within approx. 100 Å of each other, FRET occurs; there is a resulting increase in acceptor fluorescence and corresponding decrease in donor fluorescence. (**B**) Steady-state FRET between Rps2–GFP and Rps3–TCM on the 40S subunit, determined from a fluorescence emission spectrum. (**C**) Basic principle of FA. FA measures the rotational time of a labelled species in solution; a polarizing spectrophotometer measures the change in anisotropy that occurs upon species binding to become part of a larger complex. (**D**) Analysis of eIF3–SNAP–PURETIME325 binding to 40S by steady-state anisotropy (eIF3 concentration is fixed at 10 nM).



pathway. As more diverse fluors and novel tagging strategies are developed, such methods will become increasingly more important in the study of large cellular machineries more generally. Following on from recent work in the prokaryotic system, single-molecule studies of the eukaryotic translation pathway are expected to generate equally important insights. The Puglisi group has successfully immobilized bacterial ribosomes and, in combination with labelled tRNAs, has monitored tRNA dynamics within the P sites of individual ribosomes using FRET [27]. Using single-molecule fluorescence spectroscopy, time-resolved conformational changes were characterized throughout a complete cycle of translation elongation, identifying tRNA states and kinetics as they translocated from the peptidyl to the aminoacyl site within the ribosome. Steady-state FRET studies measure the dynamics of a heterogeneous mixture of molecules; as such, only average observations can be recorded. Without the 'noise' of population FRET, smFRET (single-molecule FRET) studies allow the determination of binding kinetics with unprecedented accuracy (reviewed in [29]). To achieve

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such quantitative insight into the binding kinetics of the eukaryotic translation machinery would be a remarkable feat, given the large leap in complexity. However, with the rapid development of both fluorescence techniques and technology, the first single-molecule study of the eukaryotic ribosome is expected in the near future [20].

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