smFRET studies of the ‘encounter’ complexes and subsequent intermediate states that regulate the selectivity of ligand binding

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1. Introduction

The ability of a biomolecule to selectively bind its cognate ligand when faced with the vast array of structurally similar near- and non-cognate ligands present in the cellular environment is critical for maintaining the fidelity of biological processes. Specific examples include critical processes spanning all of biology: a transcription factor binding a DNA promoter site, a small nuclear ribonucleoprotein (snRNP) binding a precursor mRNA (pre-mRNA) splice site, a microRNA binding a target mRNA, or a ribosome binding an aminoacyl-tRNA (aa-tRNA) substrate. Such specificity of ligand binding is typically achieved through multistep, ligand-binding reactions, and forming the final biomolecule–ligand complex. We note here that more complex ligand-binding reactions encompassing three or more steps allow additional opportunities for the ligand to dissociate from the post-transition state (IEC → Fig. 1) intermediate states [2]. This allows for editing mechanisms, such as kinetic proofreading [5], kinetic enhancement [6], or double-feature selection [4], all of which can greatly increase the specificity of ligand binding and selectivity of ligand-binding reactions can therefore be precisely regulated by coupling the identity of the ligand to the probability of dissociating versus the probability of proceeding along the reaction pathway. Near- and non-cognate ligands will have a higher probability of dissociating while cognate ligands will have a higher probability of proceeding along the reaction pathway and forming the final biomolecule–ligand complex. We note here that more complex ligand-binding reactions encompassing three or more steps allow additional opportunities for the ligand to dissociate from the post-transition state (IEC → Fig. 1) intermediate states [2]. This allows for editing mechanisms, such as kinetic proofreading [5], kinetic enhancement [6], or double-feature selection [4], all of which can greatly increase the specificity of ligand binding.
states to be directly observed, thereby allowing those states that were difficult to detect in ensemble biophysical experiments to be sensitively detected and comprehensively characterized [10]. Moreover, the observation of individual transitions to and from a state of interest allows heterogeneities in the physical properties of that state to be identified, sorted, and analyzed [11,12].

Despite the tremendous promise that single-molecule fluorescence approaches hold for studies of encounter complexes and subsequent intermediate states formed during ligand-binding reactions, these approaches are often limited by (i) data collection throughput that can be too low for sufficient statistical analysis, (ii) time resolutions that can be too slow to capture exceedingly transient states, (iii) limitations in spatial resolution that can make it difficult to determine when a ligand is co-localized to a biomolecule, and (iv) the ‘concentration barrier’ generated by the background fluorescence from the relatively high concentrations of fluorophore-labeled ligands that are required to observe ligand binding events within the experimental observation time that compromises the high signal-to-background ratio that is required to sensitively observe the fluorescence from a single molecule [13,14]. Notably, single-molecule fluorescence resonance energy transfer (smFRET) approaches are particularly powerful for studies of ligand-binding reactions [15–17], as they ameliorate several of these limitations, and are able to directly report on both the spatial localization as well as the conformational dynamics of biomolecules and/or ligands [18]. We begin this article by providing a brief description of the basic experimental platform that is typically used to investigate ligand-binding reactions using smFRET and then highlight two representative examples of ligand-binding reactions that have been studied using smFRET. We then close by reviewing emerging experimental and computational developments that are expanding the capabilities of smFRET experiments to characterize the encounter complexes and subsequent intermediate states that regulate the rate and specificity of ligand-binding reactions at an ever-increasing level of detail. Given the large number of ligand-binding reactions that are the subject of ongoing smFRET studies and of emerging techniques that are currently being developed, we wish to apologize to those colleagues whose work we were unable to review due to space constraints.

2. smFRET studies of ligand-binding reactions

2.1. General experimental platform

Fluorescence resonance energy transfer (FRET) is a dipole-dipole interaction in which the excitation of a donor fluorophore (e.g., Cy3) can be transferred to an acceptor fluorophore (e.g., Cy5) with an efficiency (termed the FRET efficiency, or EFRET) that, among other variables, depends monotonically on the distance between the fluorophores [19,20]. Thus, EFRET can be interpreted as a “spectroscopic ruler” (over a fluorophore pair-specific range of distances that is typically in the tens of Å) [21]. The EFRET of an energy-transfer event can be quantified by directly exciting the donor, measuring the fluorescence intensities of both fluorophores, and calculating the EFRET using the relationship

\[
\text{EFRET} = \frac{I_D}{I_A}(I_A + I_D),
\]

where \(I_A\) and \(I_D\) are the fluorescence intensities of the acceptor and the donor, respectively. Perhaps the most common experimental platform for studying ligand-binding reactions using smFRET (Fig. 2) involves tethering a biotin-derivatized, donor-labeled biomolecule of interest to the surface of a microfluidic, observation flowcell via a biotin–streptavidin–biotin bridge formed with biotin-derivatized polyethylene glycol (PEG) that has been used to functionalize the surface of the flowcell [22,23]. Introduction of an acceptor-labeled ligand into the imaging buffer within the flowcell and imaging using wide-field total internal reflection fluores-
cence (TIRF) microscopy [24–26] enables direct excitation of the surface-localized donors and simultaneous observation of both the donor- and acceptor fluorescence intensities originating from hundreds of individual biomolecules [23,27]. In such experiments, an anti-correlated donor- and acceptor fluorescence intensities versus time trajectory that exhibits single-step fluorophore photo-bleaching originating from diffraction-limited donor and acceptor spots serves as unambiguous evidence for resonance energy transfer arising from the encounter of a single, acceptor-labeled ligand with a single, surface-tethered, donor-labeled biomolecule. Important, the acceptor will only fluoresce when it is within tens of Å of the donor (i.e., when the ligand is bound to the biomolecule). Thus, smFRET enables the detection of biomolecule–ligand encounters in the presence of relatively higher, tens to one hundred nM, concentrations of fluorophore-labeled ligand in the imaging buffer than is possible with other single-molecule fluorescence microscopy approaches – thereby partially alleviating the limitations imposed by the concentration barrier described in Section 1 [28]. In addition to reporting on ligand–biomolecule encounters, smFRET can also report on conformational changes of the biomolecule and/or ligand that result in changes in the distance between the positions of the donor and acceptor. In summary, because it is able to simultaneously and sensitively report on hundreds of single biomolecule–ligand encounters at fluorophore-labeled ligand concentrations that are higher than is possible with other single-molecule fluorescence approaches, as well as because it can report on conformational changes that take place during the ligand-binding reaction, the TIRF-based smFRET experimental platform has been particularly successful for investigating the kinetic and thermodynamic properties of ligand-binding reactions [10].

2.2. aa-tRNA selection during translation elongation

In all organisms, mRNAs are translated into proteins by the ribosome, a 2.5–4.3 MDa [29,30] molecular machine that is composed of two ribonucleoprotein subunits. During translation, aa-tRNAs are delivered to the ribosomal aa-tRNA binding (A) site by the guanine triphosphatase (GTPase) elongation factor Tu (EF-Tu) as part of an EF-Tu(GTP)aa-tRNA ternary complex (TC) [31–35]. Despite the pool of 41–55 different aa-tRNA isoacceptors that is found in cells [36], the ribosome is able to accurately select the aa-tRNA whose anticodon correctly basepairs to the mRNA codon (i.e., the cognate aa-tRNA), only misincorporating aa-tRNAs with one-base mismatches (i.e., near-cognate aa-tRNAs) or with two-base or greater mismatches (i.e., non-cognate aa-tRNAs) with a frequency of 1 in $10^{3}$–$10^{4}$ [37–40]. Notably, this level of fidelity greatly exceeds the maximum misincorporation frequency of 1 in $10^{2}$ that would be expected from just the thermodynamic stability differences between the anticodon–codon interactions formed by a cognate aa-tRNA and those formed by the corresponding near-cognate aa-tRNAs [41–43]. Extensive studies of the mechanisms through which the ribosome achieves the observed high fidelity of aa-tRNA selection have led to the development of a widely accepted, multistep mechanism [44,45] in which kinetic proofreading [5,6,46–48] and induced fit [48,49] strategies

Fig. 2. Schematic of a ligand-binding reaction studied by smFRET using a typical TIRF microscope. A laser excitation source is totally internally reflected (TIR) at the interface formed between the surface of the quartz, microfluidic, observation flowcell to which the donor-labeled biomolecules are tethered and the aqueous imaging buffer in the flowcell. The evanescent field that is generated by TIR propagates into the imaging buffer and decays exponentially as a function of increasing distance from the quartz–buffer interface, thereby selectively exciting only those donors that are localized within ~300 nm of the quartz–buffer interface (top left inset). Donor and acceptor fluorescence emission is collected by an objective, separated by wavelength using dichroic mirrors, and detected using an electron-multiplying charge-coupled device (EMCCD) camera (bottom left inset). The separated donor and acceptor fluorescence intensities reporting on the binding of acceptor-labeled ligands to individual, spatially resolved, donor-labeled biomolecules can then be quantified and plotted as a function of time (bottom right inset). Figure and caption adapted from Ref. [174].
are employed to increase the otherwise relatively low fidelity of aa-tRNA selection that would be expected [33].

Over the past decade, the mechanism of aa-tRNA selection has been the subject of numerous smFRET studies [50–55]. Several aspects of the experimental systems available for studying aa-tRNA selection provide important advantages for designing, implementing, and interpreting smFRET experiments. These advantages include: (i) the involvement of interactions between only two molecular subcomplexes, the TC and a ribosomal elongation complex (REC); (ii) the availability of a reconstituted in vitro translation system composed of a full set of purified components that allows individual components and steps of the reaction to be manipulated (reviewed in Ref. [56]); (iii) the existence of a large number of small-molecule inhibitors that enable inhibition of specific and well-defined steps of the reaction [57–59]; and, (iv) the availability of a series of cryogenic electron microscopy (cryo-EM) and X-ray crystallography structures that approximate the structures of the initial and final states of the reaction, as well as those of several intermediate states (reviewed in Refs. [31,35]).

smFRET studies of aa-tRNA selection are typically performed using RECs that are biotinylated at the 5′ end of the mRNA and that have been labeled with a donor fluorophore either within the fMet-tRNA\textsuperscript{Met} that is bound at the ribosomal peptidyl-tRNA binding (P) site [50,60] or within ribosomal protein L11 [52,61]. RECs are then tethered to the surface of a microfluidic, observation flowcell via their 5′-biotinylated mRNA such that they can be imaged with single-molecule resolution using TIRF microscopy. Stopped-flow delivery of a TC carrying an acceptor-labeled aa-tRNA to an REC carrying a donor-labeled P-site tRNA and a cognate A-site codon then yields acceptor- and donor intensities versus time trajectories that are used to calculate pre-steady-state EF\textsubscript{RET} versus time trajectories. EF\textsubscript{RET} versus time trajectories initiate at zero-EF\textsubscript{RET} and evolve through transiently sampled low- and mid-EF\textsubscript{RET} states before arriving at a high-EF\textsubscript{RET} final state that is consistent with structures approximating the final state of the reaction in which the aa-tRNA has been accommodated into the A site (Fig. 3A) [50–55]. The mid-EF\textsubscript{RET} state has been assigned to a mixture of at least two intermediate states that had been previously observed in biochemical [48] and structural studies [62,63], and that correspond to the conformations of the TC-bound REC that immediately precede and immediately follow ribosome-catalyzed GTP hydrolysis by EF-Tu (Fig. 3A) [50]. The state that precedes GTP hydrolysis can be biochemically ‘captured’ and stabilized using a non-hydrolyzable GTP analog [64] or a GTP hydrolysis-deficient EF-Tu mutant [65]. Likewise, the state that immediately follows GTP hydrolysis can be captured and stabilized using the EF-Tu-targeting antibiotic kirromycin [66]. Such approaches allow the populations of these ordinarily transient and low-population states to be increased such that they can be easily studied using ensemble biochemical and structural methods [31–33,35].

In contrast to the mid-FRET state, the low-EF\textsubscript{RET} state has been assigned to a structurally novel intermediate state that has thus far eluded capture and stabilization using mutations, biochemical analogs, or small-molecule inhibitors, thereby precluding its direct detection using ensemble biochemical or structural studies (Fig. 3A). Nonetheless, the conformation of the TC-bound REC corresponding to the low-EF\textsubscript{RET} state is a critical, codon-dependent intermediate state during aa-tRNA selection. Experiments in which the
TC is delivered to an REC carrying a non-cognate A-site codon, for example, do not result in any detectable smFRET signals, including even the detection of highly transient sampling of the low-EFRET state. Analogous experiments using RECs carrying a near-cognate A-site codon, on the other hand, result in the detection of a highly transient low-EFRET state that corresponds to the formation of a weakly interacting, transient TC-bound REC from which TC has a much higher probability of dissociating from the REC than of progressing along the reaction pathway. In contrast, experiments using RECs carrying a cognate A-site codon result the detection of a slightly longer-lived low-EFRET state that corresponds to the formation of a slightly more stably interacting, less transient TC-bound REC from which the TC has a much higher probability of progressing along the reaction pathway than of dissociating from the REC. Interestingly, the precise EFRET values of the low-EFRET state differ for cognate and near-cognate TC-bound RECs, indicating that the TC in a cognate TC-bound REC is positioned in a manner that differs slightly from how it is positioned in a near-cognate TC-bound REC. This suggests that positioning of the TC within the low-EFRET TC-bound REC state is an important, yet transient, structural response to the recognition of a cognate codon at the A site of the REC.

Despite the success of smFRET in identifying and characterizing the highly transient low-EFRET TC-bound REC state, it is important to note that there are one or more states preceding this state in the reaction pathway that play important roles during aa-tRNA selection and that remain to be identified and characterized. For example, the very first state resulting from the codon-independent, initial binding of the TC to the REC, which is likely to have properties that are similar or identical to those of an encounter complex, has yet to be observed (Fig. 3A). In addition, the fact that the low-EFRET state is not detected in smFRET experiments in which a TC is delivered to an REC carrying a non-cognate A-site codon suggests that, either: (i) the low-EFRET State is sampled too rarely to be detected due to current experimental limitations (e.g., the low concentrations of acceptor-labeled TC that must be used to maintain the signal-to-background required to observe single fluorophores, the rate with which the donors on the RECs photobleach, etc.); (ii) the low-EFRET state is sampled too transiently to be detected due to current limitations on the time resolution of the experiments; (iii) non-cognate TCs are recognized and discriminated against within a state that precedes and is physically distinct from the low-EFRET state, but in which the distance between the donors and acceptors in the currently available fluorophore labeling schemes are too far away to generate a detectable EFRET; or (iv) a combination of these possibilities. Thus, weakly interacting, highly transient states that are critical for fully understanding the physical and molecular mechanisms underlying aa-tRNA selection during translation remain to be identified and characterized – representing an ongoing challenge for the field.

Towards these goals, emerging technologies hold great promise for ongoing investigations of the mechanism of aa-tRNA selection. Recent single-molecule fluorescence co-localization microscopy experiments that use next-generation, nanofabricated, microfluidic, observation flowcells to overcome the concentration barrier (see Section 3.3), for example, have demonstrated that fluorophore-labeled TCs do indeed transiently co-localize with fluorophore-labeled RECs carrying non-cognate A-site codons. Although these were not smFRET experiments in which the measured EFRET could be compared to what is observed in RECs carrying near-cognate or cognate A-site codons, they represent an important step towards understanding where in the pathway and how RECs discriminate against non-cognate TCs [67].

2.3. Splice site selection during spliceosome assembly

In eukaryotes, transcription of the genomic DNA often produces pre-mRNAs that must be spliced in order to excise the non-coding intronic sequences and ligate the coding exonic sequences, ultimately yielding mature mRNAs that can be translated by the ribosome. Pre-mRNA splicing is carried out by the spliceosome, a 2–3 MDA molecular machine that is composed of five RNA-protein complexes known as snRNP complexes and dozens of additional protein factors (Fig. 3B) [68–72]. During splicing, the spliceosome must assemble de novo across each pre-mRNA intron. Assembly often begins with binding of the U1 snRNP to the junction of the intron with the 5’ exon (i.e., the 5’ splice site) and one or more proteins to the short sequence that specifies the 2’-OH for nucleophilic attack of the phosophodiester bond at the 5’ splice site during the first chemical step of splicing (i.e., the branchsite). Some of these branchsite-binding proteins (Branchpoint Bridging Protein in yeast or SF1 in humans) are then displaced by binding of the U2 snRNP. This is then followed by binding of the U4/U6.U5 tri-snRNP and the protein-only nineteen complex (Ntc), an event that ultimately triggers the release of the U1 and U4 snRNPs and activates the spliceosome for catalysis [68,69,72,73]. Despite the fact that the 5’ splice site, the junction of the intron with the 3’ exon (i.e., the 3’ splice site), and the branchsite are comprised of short consensus sequence elements that, at least in metazoans, are very poorly conserved [73–75], the spliceosome is able to accurately recognize and select these sequence elements in order to generate the proper mRNA with an error rate for splicing of 1 in 10^7 to only 1 in 10^10 [73,76–78]. Similar to aa-tRNA selection, the unexpectedly high fidelity of splice site selection by the spliceosome has been attributed to multistep mechanisms for spliceosome assembly, activation, and catalysis in which kinetic proofreading [5,6] strategies are employed to increase the low fidelity of splice site selection that would otherwise be predicted [73,75].

Although studies of splice site selection during spliceosome assembly would ideally be performed using smFRET [69], the experimental systems available for studying splice-site selection during spliceosome assembly present several important challenges for designing, implementing, and interpreting smFRET experiments. These challenges include, (i) the fact that even a minimal version of the reaction involves the interaction of numerous molecular subcomplexes; (ii) the unavailability of a reconstituted version of the spliceosome for catalysis [68,69,72,73]; (iii) the existence of only a few small-molecule inhibitors that enable inhibition of specific and well-defined steps of the reaction [79]; (iii) the existence of only a few small-molecule inhibitors that enable inhibition of specific and well-defined steps of the reaction [79]; and (iv) the availability of only a very limited number of cryo-EM and/or X-ray crystallography structures (reviewed in Ref. [82]) for designing donor–acceptor labeling schemes. Due to these challenges, few smFRET studies of the spliceosome have been performed, and these either focus on structural rearrangements of a donor- and acceptor-labeled pre-mRNA upon binding of the indirectly detected, fluorophore-free spliceosomal components [83,84] or on structural rearrangements of protein-free, RNA components of the snRNPs [85,86]. Notably, smFRET studies reporting on the dynamics of spliceosome assembly and how these dynamics are modulated by the identities of the 5’- and 3’ splice sites and the branch point sequence have not been reported. Recently, however, a combination of chemical biology approaches for systematically and orthogonally fluorophore-labeling sets of spliceosomal subcomplexes within Saccharomyces cerevisiae (S. cerevisiae) whole cell extracts (WCEs) and single-molecule fluorescence co-localization microscopy experiments have been used to investigate the dynamics of spliceosome assembly. Notably, this approach enabled the authors to demonstrate how these dynamics are modulated by the unique splice-site features of specific pre-mRNA introns [79,84].

WCEs for single-molecule fluorescence co-localization microscopy experiments were prepared from S. cerevisiae strains in which
the C-termini of specific proteins that are associated with sets of well-defined spliceosomal subcomplexes had been fused to either a ‘DHFR’ tag (a variant of *Escherichia coli* dihydrofolate reductase) or a ‘SNAP’ tag (a variant of human alkylglycine S-transferase) [79,84]. DHFR- and SNAP fusions are then differentially fluorophore labeled using fluorophore-labeled trimethoprim analogs that bind DHFR very tightly, with an equilibrium dissociation constant of ~1 nM [87], or benzyl-guanine derivatives that covalently modify the active site cysteine of SNAP [88]. Single intron-containing pre-mRNAs to be used for single-molecule fluorescence colocalization microscopy were fluorophore labeled near the 3' end, biotinylated at the 3' end, and then tethered to the surface of a microfluidic, observation flowcell via their 3' biotin. In this manner, they could be imaged using TIRF microscopy and their spatial location in the field-of-view could be determined to within single-molecule resolution [89]. Introduction of a WCE containing a set of fluorophore-labeled spliceosomal subcomplexes into such a flowcell then allows binding of each subcomplex to the fluorophore-labeled and surface-tethered pre-mRNA to be monitored via their spatial co-localization with the pre-mRNA.

Collectively, these studies have revealed that, depending on the identity of the pre-mRNA, spliceosome assembly preferentially follows one of two pathways that differ in the order in which the U1 and U2 snRNPs are observed to co-localize to the pre-mRNA (Fig. 3B). Thus, the first steps of the assembly reaction may be either U1 → U2 → U4/U6.U5 → NTC or U2 → U1 → U4/U6.U5 → NTC. In addition, these studies revealed that no single step is rate limiting for the overall assembly reaction. Perhaps most interesting, these experiments have demonstrated that, for all pre-mRNAs tested thus far, binding of each subcomplex is highly reversible and transient on the timescale of the experiments. This result suggests that introns are not irreversibly committed to splicing during the early steps of spliceosome assembly. Instead, commitment to splicing increases with the binding of each successive subcomplex and can possibly be regulated by modulating the stabilities of the bound subcomplexes during spliceosome assembly. Indeed, the set of pre-mRNAs that have been tested thus far differ in, among other respects, the sequences of their 5' and 3' splice sites, suggesting that differences in the order in which the U1 and U2 snRNPs are observed to co-localize to the different pre-mRNAs may arise from pre-mRNA sequence-dependent differences in the stabilities with which U1 and/or U2 snRNPs bind to each pre-mRNA. Consistent with this possibility, more extensive scrambling of the 5' splice site results in a loss of detectable U1 snRNP binding in both ensemble [90–92] and single-molecule fluorescence [79] experiments, strongly suggesting that the stability of U1 snRNP binding is highly dependent on the identity of the 5' splice site.

Despite the success of the single-molecule fluorescence colocalization experiments described in the previous paragraph, additional studies in which the 5' splice site, the 3' splice site, and the branch point sequence are systematically mutagenized will be needed to determine how the transient binding of individual subcomplexes during spliceosome assembly across introns is modulated. In addition, the fact that extensive scrambling of the 5' splice site results in a loss of detectable U1 snRNP binding to the 5' splice site suggests that the encounter complex between U1 snRNP and the pre-mRNA and, possibly, subsequent U1 snRNP-bound pre-mRNA intermediate states during which U1 snRNP recognizes and selects the 5' splice site, have yet to be observed. Ultimately, it will be important to extend these types of experiments to investigate recognition and selection of the 3' splice site by a subunit of the U2 auxiliary factor (U2AF) [102–104]) and the branchsite sequence by SF1 and U2 snRNP [105] during the early steps of spliceosome assembly. Thus, numerous, weakly interacting, highly transient states that are critical for fully understanding splice site selection during spliceosome assembly remain to be identified and characterized, representing an ongoing challenge for the field.

### 3. Emerging experimental advances for smFRET studies of ligand-binding reactions

#### 3.1. Microscopy developments

As described in Section 1, one of the current limitations in single-molecule fluorescence studies of ligand-binding reactions is the concentration barrier that is created by the relatively high concentrations of fluorophore-labeled ligands that are required to be present in the imaging buffer in order to detect ligand-binding events within the experimental observation time [14]. Because an acceptor will only be efficiently excited via FRET when it is within tens of Å of a donor that is directly excited by an excitation light source (e.g., a laser), smFRET experiments in which a donor-labeled biomolecule of interest is tethered to the surface of the microfluidic, observation flowcell and an acceptor-labeled ligand is supplied in the imaging buffer greatly ameliorate the limitations imposed by the concentration barrier [28,106]. Despite this advantage of smFRET experiments, acceptors can still be directly, albeit inefficiently, excited by the laser that is used to directly excite the donor fluorophore – a low probability event known as excitation crosstalk [10]. At high enough concentrations of acceptor-labeled ligands in the imaging buffer (typically well below the equilibrium dissociation constants of weakly interacting biomolecule–ligand complexes), excitation crosstalk becomes a major source of background fluorescence noise in such experiments, a situation that can ultimately prevent the detection of fluorescence from individual molecules [14].

By preventing the excitation source from penetrating deeper than ~200–300 nm into the imaging buffer in the flowcell, wide-field TIRF microscopy-based smFRET experiments minimize the total amount of noise from excitation crosstalk and time resolution of using wide-field smFRET to study encounter complexes. Using a confocal microscope with an avalanche photodiode (APD) or a single-photon avalanche diode (SPAD) detector, rather than a TIRF microscope with an EMCCD camera that are used as detectors in TIRF microscopy-based smFRET experiments operate at time resolutions that are typically too slow to capture the weakest and most transient intermediate states (typically limited to ~30 ms per a full-resolution frame [107]). This tradeoff between lower background noise and faster time resolution is a fundamental limitation of using wide-field smFRET to study encounter complexes. Using a confocal microscope with an avalanche photodiode (APD) or a single-photon avalanche diode (SPAD) detector, a TIRF microscope with an EMCCD camera detector, affords significant increases in the sensitivity, signal-to-background ratio, and time resolution (typically ~1 ms per data point) of smFRET experiments [10,108]. Unfortunately, however, this increase in the time resolution comes at the cost of a significant decrease in throughput, as the confocal fluorescence microscopy-based approach can only image one biomolecule at a time, whereas the TIRF microscopy-based approach can simultaneously image hundreds of surface-tethered biomolecules at a time. Such low throughput can be particularly difficult to overcome when attempting to detect weakly interacting, transient biomolecule–ligand complexes and/or conformational states that are rarely populated. In the ideal setup, one would be able to couple a TIRF...
microscope with an APD- or SPAD-array detector [107,109]. Such an approach would allow wide-field detection with APDs or SPADs, thereby allowing the high throughput associated with using a conventional TIRF microscope, but with the high time resolution that is associated with using a confocal fluorescence microscope. Towards this goal, smFRET was recently demonstrated using linear arrays of 8 SPADs [110], and so, with further development, commercially available products composed of tens of thousands of SPADs will likely constitute an important future development in the field of single-molecule fluorescence microscopy [109].

An alternative approach to minimizing the excitation crosstalk of acceptor-labeled ligands and improving the signal-to-background ratio of conventional TIRF microscopy-based smFRET experiments without compromising throughput is to remove excess ligand from the excitation volume without changing the local concentration of the ligand in the vicinity of the biomolecule. This can be achieved by locally confining ligands and the biomolecule of interest into spatially resolved regions of the flowcell that are smaller than the excitation volume associated with the detection of single, diffraction-limited spots in the field-of-view. Notable approaches include electrostatic and physical traps [111,112] as well as encapsulation of the biomolecules and ligands in aqueous drops in oil [113]. Perhaps the most promising and physiologically compatible method of local confinement for smFRET experiments is to encapsulate ligands and individual biomolecules in surface-tethered liposomes [114]. These lipid vesicles not only encapsulate ligands with individual biomolecules and confine them to spatially resolved regions on the surface of the microfluidic flowcell, but, because the biomolecule is free to diffuse within the surface-tethered liposome, they also serve to further reduce the interactions that a biomolecule of interest might have with the PEG-passivated surface of the microfluidic flowcell [115]. In addition, since their introduction in 2001 [116], liposomes have been optimized to allow for the free diffusion of small molecules into and out of the liposome [117].

3.2. Fluorophore developments

Although much work has gone into developing robust donor and acceptor pairs with well-behaved photophysics for smFRET studies [118], photobleaching and ‘blinking’ of fluorophores continues to impose limitations on smFRET studies of biochemical systems [119,120]. The irreversible photobleaching of a fluorophore effectively brings the smFRET experiment, at least for that fluorophore, to an end, thereby limiting the experimental throughput. The reversible blinking of a fluorophore similarly limits the throughput of smFRET experiments and, in addition, can convolute the subsequent analysis of the data (e.g., it is often difficult to distinguish whether changes in $E_{\text{FRET}}$ that are observed arise from bone fide changes in the distance between the donor and acceptor or from blinking of the donor or acceptor) [119,120]. The limitations imposed by photobleaching and blinking are particularly challenging for smFRET studies of ligand-binding reactions. Rare events such as the formation of a particularly long-lived complex formed by the generally weak, transient binding of an acceptor-labeled ligand to a surface-tethered, donor-labeled biomolecule of interest, for example, are exceedingly difficult to observe within the limited observation times that are imposed by photobleaching and blinking of the donor fluorophore. The continued development of longer-lived, brighter, and more stable fluorophores, such as Cy3B [121], is therefore an important area of research, as these enhanced fluorophores enable longer and more stable observation times in smFRET experiments [118,122].

Complementing the development of longer-lived, brighter, and more stable fluorophores, considerable work has also gone into optimizing buffer conditions that minimize photobleaching and blinking [119]. Often, photobleaching is mediated via a photochemical reaction between a fluorophore in its electronically excited state and molecular oxygen. As a consequence, oxygen scavenging systems, including mixtures of glucose, glucose oxidase, and catalase or of protocatechatic acid (PCA) and protocatechuate-3,4-dioxygenase (PCD), are often added to experimental buffer systems to extend fluorophore survival times [123,124]. As early as 1988, β-mercaptoethanol (BME) was being added to buffer systems for single-molecule fluorescence microscopy experiments in order to suppress the blinking of tetramethylrhodamine, presumably by quenching a dark triplet-state of the fluorophore [123]. Since then, triplet-state quenchers, including mixtures of cyclooctatetraene (COT) [23], 4-nitrobenzyl alcohol (NBA) [23], and/or Trolox (a water-soluble analog of vitamin E) [125] have become standard additives in smFRET experiments [126]. Recently, direct, covalent conjugation of triplet-state quenchers such as COT, NBA, or Trolox to the entire class of cyanine dye fluorophores, including Cy3 and Cy5, was shown to significantly enhance the photostability of these fluorophores [127,128]. Although these new fluorophore–quencher conjugates represent a breakthrough in fluorophore development, caution should be exercised in their use, as these conjugates are necessarily larger than the traditional fluorophores on which they are based and are therefore more likely to sterically perturb the biomolecules or ligands to which they are attached.

In addition to the development of fluorophores and/or buffer conditions that resist photobleaching and blinking, the development of fluorophores that enable selective excitation is another promising area of current research [120]. Indeed, one of the major advantages of smFRET is that the acceptor is excited selectively (i.e., only when it is within tens of Å of a donor). Similarly, highly enzyme-specific fluorogenic substrates that fluoresce exclusively upon being enzymatically modified can be used to conduct single-molecule fluorescence microscopy studies of the mechanisms of action of enzymes for which fluorogenic substrates have been developed [12,122]. This strategy, however, is difficult to generalize to all, or even many, enzymes and even more so to non-enzymatic biomolecules. In addition, to our knowledge, fluorogenic strategies have not yet been utilized in smFRET studies. As an alternative to the use of fluorogenic substrates, selective excitation can be achieved through the use of photoswitchable fluorophores [120]. For example, in the PhADE technique, an imaging buffer containing high concentrations of a photoswitchable fluorophore-labeled ligand is activated with a laser pulse and these are allowed to diffuse out of the imaging volume. This effectively removes all of the photoactivated ligands other than those that are bound to the surface-tethered biomolecules from the flowcell, thereby removing much of the background fluorescence that would otherwise arise from photoactivated ligands in solution [129]. Unfortunately, this strategy works only for relatively long-lived, weakly interacting biomolecule–ligand complexes, and, to our knowledge, has not yet been used in smFRET experiments.

Perhaps the most promising recent development in smFRET studies of biomolecular systems is the use of FRET-based quenchers as acceptors for smFRET experiments [130]. By returning from the excited state to the ground state without emitting a photon, quenchers not only free the optical spectrum for the use of additional fluorophores (e.g., for colocalization experiments), but, of particular importance for smFRET studies of ligand-binding reactions, also allow smFRET experiments to be performed under conditions in which very high concentrations of quencher-labeled ligands can be included in the imaging buffer without significantly increasing the fluorescence background. This is because a quencher that is excited by excitation crosstalk will not fluoresce, thereby minimizing the contribution of excitation crosstalk to the fluorescence background. Only a few researchers have thus far used FRET-
based quenchers to address biological systems using smFRET [130–134]. Notably, a FRET-based quencher was used to report on the large-scale conformational dynamics of the ribosome during translation elongation [135,136], although this approach was used to free the optical spectrum and expand the number of fluorophores that could be simultaneously detected, rather than to use FRET-based quencher-labeled ligands to overcome the concentration barrier.

3.3. Optical engineering developments

A general strategy for increasing the sensitivity of single-molecule fluorescence experiments is to enhance the fluorescence signal from the molecules of interest. Perhaps the most widely used approach for accomplishing this is plasmon-mediated enhancement, which has traditionally been used to enhance optical spectroscopies (e.g., surface plasmon-enhanced Raman spectroscopy) and, in single-molecule fluorescence applications, can be used to decrease illumination volumes and increase fluorescence signals [137]. Nanofabricated plasmonic bowties [138] and plasmonic nanoantennas [139–141] have both been shown to dramatically increase signal-to-background ratios in single-molecule fluorescence applications. Of particular importance for single-molecule fluorescence studies of ligand-binding reactions, the ‘antenna-in-a-box’ platform has used plasmon-based enhancement to permit the observation of single-molecule fluorescence in the presence of micromolar concentrations of fluorophore in the imaging buffer [142]. Unfortunately, few of these platforms have thus far demonstrated their applicability with smFRET [139] and, while these technologies show promise, the fact that highly specialized facilities, equipment, and technical skills are required to nanofabricate the plasmon-producing structures (e.g., the bowties and antennas) onto the surface of the microfluidic flowcells that are used in single-molecule fluorescence experiments has thus far hindered widespread adoption by the community.

Another general strategy for attaining higher signal-to-background ratios in single-molecule fluorescence studies is to decrease the fluorescence background by reducing the excitation volume. For this purpose, arrays of nanoapertures – nanoscopic wells that have been fabricated into a thin metallic layer that has been deposited onto the surface of a microfluidic flowcell (Fig. 4A) – have proven themselves very useful. Popularized as “zero-mode waveguides” (ZMW), the geometric confinement of light in the nanoaperture leads to a zeptoliter excitation volume at the very bottom of the nanoaperture. This permits the sensitive observation of a single, fluorophore-labeled molecule that has been tethered or otherwise localized to the bottom of the nanoaperture in a background of micromolar concentrations of fluorophore-labeled molecules in the imaging buffer [143]. Nanoaperture arrays are used in Pacific Biosciences’ next-generation, single-molecule real-time (SMRT) DNA sequencing technology (http://www.pacificbiosciences.com). There, a single DNA polymerase is tethered to the bottom of each nanoaperture, and a nucleotide-specific, single-molecule fluorescence signal is observed as the polymerase incorporates each nucleotide into the nascent DNA molecule that is being synthesized [144].

Unfortunately, in the more than ten years since nanoaperture arrays were first introduced for single-molecule fluorescence applications [143], only a handful of biomolecular systems beyond DNA replication have been investigated using this technology [67,136,145–147]. Notably, studies of all but one of these biomolecular systems have been investigated in collaboration with Pacific Biosciences and only one of these studies has used nanoaperture arrays in an smFRET application. As is the case with plasmon-based enhancement technologies, widespread adoption of nanoaperture-array technology has likely been hindered by the limited availability of the resources required for nanofabrication. In addition, the non-specific adsorption of biomolecules to the metallic and glass surfaces of the nanoapertures has likely limited their applications. Non-specific adsorption not only alters working concentrations by sequestering molecules from solution, but it also compromises single-molecule resolution by non-specifically localizing multiple, fluorophore-labeled molecules to the surfaces near the bottom of the nanoapertures. In the case of SMRT sequencing via DNA replication, a very successful polyvinyl phosphonic acid-based, nanoaperture-surface passivation scheme was developed that minimizes the non-specific adsorption of small, negatively charged nucleotide triphosphates to the surface of aluminum-based nanoapertures.
Given the negatively charged nature of the polyvinyl phosphonic acid passivation layer, however, it is likely that this passivation scheme will not be generally applicable to other biomolecules (e.g., positively charged globular proteins).

More recently, an alternative passivation scheme has been reported that uses thiol-based self-assembled monolayers (SAMs) of PEG to robustly passivate gold-based nanoapertures [106]. Given the widespread success of PEG-based passivation schemes in single-molecule fluorescence studies, we anticipate that PEG-passivated nanoaperture arrays will provide a more general solution to the problem of non-specific adsorption of biomolecules to the nanoaperture surfaces and will thereby enable studies of a wide-range of biomolecular systems using nanoaperture arrays (Fig. 4A). Perhaps most excitingly, the use of gold-based nanoapertures such as the PEG-passivated, gold based nanoapertures described by Kinz-Thompson et al. [106] also allows the surface-plasmon of the gold to be used for plasmon-based enhancement of the fluorescence signals at the bottom of the nanoapertures, an approach that has been recently demonstrated [149,150] and that could potentially facilitate the use of nanoaperture arrays for smFRET applications.

4. Emerging computational advances for smFRET studies of ligand-binding reactions

4.1. Review of single-molecule kinetics

In order to discuss recent improvements in the analysis of smFRET data, it is useful to begin with a brief, general review of how kinetic information is obtained from single-molecule experiments and, more specifically, smFRET experiments. Traditional treatments of chemical kinetics are insufficient to describe systems where fluctuations from the average behavior are important, such as systems with small numbers of molecules [151]. Instead, alternative approaches that treat molecules as independent, stochastic entities are used [152]. To interpret the kinetic data yielded by a single molecule from a smFRET experiment, a framework is used which describes the distribution of times that the molecule spends dwelling in a particular state [151–157]. To extract these dwell time distributions from single-molecule $E_{\text{FRET}}$ versus time trajectories, dwell-time histograms are built from the results of analyzing the data using methods, such as hidden Markov models (HMM) (see Section 4.2, below), change point analysis, or wavelet analysis [158], that detect the time points at which switching between distinct $E_{\text{FRET}}$ states occurs. It is worth noting that a complete theoretical description has been developed that allows high-resolution information about the conformational dynamics of a biomolecular system to be extracted from the joint distribution of $E_{\text{FRET}}$ values and fluorescence lifetimes determined from experimentally observed photon bursts [159,160]. Although the joint distribution provides more information regarding the conformational dynamics of the biomolecular system than the distribution of $E_{\text{FRET}}$ values alone, this approach is not generally applicable in traditional, wide-field, TIRF microscopy-based smFRET experiments, as these experiments do not monitor photon arrival times.

4.2. Hidden Markov model developments

One of the most popular approaches to analyzing single-molecule $E_{\text{FRET}}$ trajectories has been the application of hidden Markov models (HMMs), which yield the probabilities of transitioning between states as well the ‘idealized’ path between states. HMMs were first used in biology for analyzing conductance time series in single channel ion recordings [161], and first suggested for use with smFRET data in 2003 [162]. Since then, a number of software packages for HMM analysis of smFRET data have been published [163–168]. Although HMM analysis of smFRET data can provide the desired information necessary to develop mechanistic models of biomolecular function, HMM analysis approaches and software packages that use maximum likelihood methods to estimate HMM parameters (e.g., HaMMy [163], QuB [169], and SMART [164]) can result in overfitting of the smFRET data (i.e., overestimating the number of states that can be confidently ascribed to the data), as the value of the likelihood function that is being maximized will always increase with the number of states included in the analysis. This overfitting problem has been recently addressed by HMM analysis approaches and software packages that use Bayesian inference methods to estimate HMMs (e.g., vbFRET [165,166] and ebFRET [167,168]). Despite the success of HMMs for the analysis of smFRET data, however, it is important to note that HMMs are ill-suited for modeling $E_{\text{FRET}}$ trajectories containing rapid transitions such as those into and out of energetically unstable, transiently sampled states (e.g., weakly interacting biomolecule–ligand complexes with rapid rates of association and dissociation) and are inappropriate for modeling non-Markovian data (e.g., data exhibiting ‘dynamic disorder’ where the probability of a transition changes with time) [165–168].

4.3. Bayesian inference developments

Perhaps the most promising computational approaches for analyzing smFRET data from weakly interacting, transient biomolecule–ligand complexes employ Bayesian inference, and in doing so they are able to ‘learn’ from the data [170]. Bayesian inference approaches have been used in the analysis of smFRET data by removing noise from $E_{\text{FRET}}$ trajectories [171] and by analyzing photon bursts [172,173]. Perhaps the most powerful applications, however, are those that use Bayesian inference methods to estimate the HMM parameters that are used to analyze $E_{\text{FRET}}$ trajectories [165–168]. The vbFRET software package, for example, uses Bayesian inference on an HMM to select the number of states and rates of transitions between states (i.e., the kinetic model) that best describes each individual $E_{\text{FRET}}$ trajectory. This minimizes the overfitting problem faced by approaches and software packages that use maximum likelihood methods to estimate HMMs and subsequently rely on the user or on an ad hoc metric, such as the Bayesian- or Akaike information criteria, to select the kinetic model that best describes the data [165,170]. Moreover, vbFRET shows promise at detecting transiently sampled intermediate states that maximum likelihood approaches for estimating HMMs might otherwise miss, an extremely useful ability when studying weakly interacting, transient biomolecule–ligand complexes using smFRET [165].

One of the major limitations of all HMM-based approaches for smFRET data analysis is that these approaches provide a separate and unique kinetic model for each individual $E_{\text{FRET}}$ trajectory. Ultimately, it is left up to the user to determine which single, consensus kinetic model best describes the entire population of kinetic models provided by the HMM-based analysis of the ensemble of $E_{\text{FRET}}$ trajectories that are typically obtained from a single smFRET experiment. Recently, van de Meent and coworkers have addressed this problem by expanding on the vbFRET framework and creating ebFRET. ebFRET uses a Bayesian inference method to estimate HMM parameters that is analogous to the one that vbFRET uses, but does so on entire populations of $E_{\text{FRET}}$ trajectories rather than on individual $E_{\text{FRET}}$ trajectories, thereby using all of the $E_{\text{FRET}}$ trajectories to learn the single, consensus kinetic model that best describes all of the available data (Fig. 4B) [167,168]. Because it uses all of the $E_{\text{FRET}}$ trajectories, ebFRET has an enhanced and statistically robust ability to detect rarely and transiently sampled states that might appear in some, but not all, of the $E_{\text{FRET}}$ trajectories associated with a single smFRET experiment. In addition,
ebFRET can distinguish between states that have the same \( E_{FRET} \) but differ in their lifetimes. For example, ebFRET has been used to distinguish between two structurally similar states of a biomolecule that yield the same \( E_{FRET} \) but that differ in the lifetime of that state due to the presence or absence of a bound ligand [168] – a development that dramatically extends the resolution of smFRET experiments.

One of the major challenges for HMM analyses of data collected from smFRET studies of weakly interacting, transient biomolecule–ligand complexes is that the encounter complexes and many of the intermediate states that are sampled are simply too energetically unstable and too transiently sampled to be reliably modeled with a HMM [165]. This is only likely to become more challenging as ever more sensitive and higher time resolution single-molecule biophysical techniques are developed that increasingly render the most weakly interacting and transient biomolecule–ligand complexes accessible to study. Recently, however, a non-HMM-based Bayesian inference approach called BIASD has been developed that, assuming a two-state model (e.g., the ligand-bound and ligand-free states of a biomolecule), is able to learn both the \( E_{FRET} \) values and rates of transitions into and out of transiently sampled states (e.g., the rates of ligand association and dissociation from a biomolecule) in cases where the rates of transitions are on the order of, or even faster than, the experimental time resolution [Kinz-Thompson in preparation]. This method should therefore prove extremely useful in ongoing efforts to study weakly interacting, transient biomolecule–ligand complexes using smFRET.

5. Conclusion

While space constraints necessarily limited the focus of the present article to intermolecular ligand-binding reactions, we note that many of the concepts, experimental techniques, and computational approaches discussed in this article apply equally well to studies of the weakly interacting, transient intermediate states that are sampled during intramolecular biomolecular folding reactions and intramolecular structural rearrangements that are associated with function. Regardless, understanding the origins of specificity in ligand-binding reactions is at the core of many research investigations and, as such, there is a great need to develop technologies that enable such reactions to be investigated at an ever increasing level of detail, a need that single-molecule fluorescence and, specifically, smFRET approaches are uniquely positioned to address.

By initially forming an encounter complex with a potential ligand and subsequently proceeding to form other weakly interacting, transient intermediate states prior to forming a final, stably bound biomolecule–ligand complex, biomolecules can rapidly and efficiently screen potential ligands and control the specificity of binding during ligand-binding reactions. Although space constraints limited us to highlighting only two, aa-tRNA selection by the ribosome and splice site selection during splicing assembly, there are now many examples of ligand-binding reactions that are being actively investigated using single-molecule fluorescence colocalization approaches and, at higher resolution, using smFRET approaches [15–17].

Despite the promise and success of smFRET approaches for overcoming these challenges, will enable encounter complexes and other weakly interacting, transient intermediate states to be investigated at an ever increasing level of detail. Such studies promise to reveal the mechanisms that control the selectivity of ligand-binding reactions at unprecedented resolution – knowledge that will allow us not only to understand, but also to modulate, some of the most important processes in all of biology.

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