distinct substrates. Although the processing kinetics of individual substrates have been examined, comparison of processing kinetics and kinetic competition between substrates has not been explored. To understand the basis for multiple substrate recognition by the holoenzyme we determined kinetic schemes forpre-tRNA608MET, a consensus pre-tRNA, and pretRNA605fmet, a non-consensus pre-tRNA, using fluorescence assays and standard discontinuous assays. Remarkably, the results show processing kinetics to be uniform with the rate limiting step being association. To obtain a complete and comprehensive understanding of how sequence and structure influence processing rates and competition between substrates, we undertook two complementary directions. First, we directly examined the competition between pre-tRNA608MET and pre-tRNA605fmet distinguishing products using different leader sequence length. Second, we designed a high throughput method to determine the effects, if any, of differences in pre-tRNA leader sequence and structure by using deep sequencing to follow the range in distribution of different substrate sequence variants in the same reaction.

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Single Molecule FRET Characterization of Pre-mRNA Splicing: Substrate Dynamics during Recognition and Catalysis

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The spliceosome is a complex ribonucleoprotein (RNP) that catalyzes the removal of intervening sequences (introns) from coding regions (exons) in eukaryotic pre-mRNAs. The ordered assembly of the spliceosome makes it a compositionally dynamic system where protein and RNA components are shuttled in and out in a highly regulated manner. Furthermore, catalytic activation of the spliceosome requires a dynamic set of ATP dependent RNA-RNA and RNA-protein interactions. Extensive genetic and *in vitro* biochemical work has been done to define the *cis*- and *trans*- acting factors that are required for both steps splicing. On the other hand, the pre-mRNA substrate which acts as both the scaffold for assembly and is a key component of the chemical steps after activation by a yet to be identified spliceosomal catalyst has been wholly ignored thus far. The difficulty in interrogating this system without synchrony and inactivation, thus precluding real time measurements, has proven difficult to overcome. To dissect the kinetic and conformational requirements for pre-mRNA positioning during spliceosome assembly we developed an *in vitro* single molecule FRET (smFRET) splicing assay in which the position of conserved intronic sequences have been tracked in real-time throughout splicing. The ability to stall the splicing process with mutations in either the pre-mRNA or splicing components we can dissect the role of spliceosome components in regulating pre-mRNA conformations. Additionally, we have utilized an affinity purification technique to determine the relative positions of conserved sequences in isolated splicing intermediates. We have observed reversible conformations changes whose kinetics are affected by ATP and the identity of splice sites.The kinetics of these conformations has implications for proofreading and catalysis that are essential for the maintenance of proper gene expression.

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tRNA Annealing onto the HIV-1 Genome Studied by FRET Spectroscopy and Microscopy

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In the HIV life cycle, reverse transcription allows conversion of a single stranded genomic RNA into double stranded DNA, capable of integration. A prerequisite for the reverse transcription process is the formation of the initiation complex between the RNA genome and a host-cell tRNA that is used as a primer by reverse transcriptase. The nucleocapsid protein (NC) is involved in this process, which requires hybridization of the tRNA to the complementary primer binding site (PBS) sequence on the RNA genome. Besides the tRNA-PBS interaction, other interactions were proposed to play a role such as an 8-nucleotide motif in the U5 region of the untranslated leader RNA, named the primer activation signal (PAS). We investigate the tRNA annealing process and the presence of a tRNA-PAS interaction using fluorescence resonance energy transfer (FRET) spectroscopy and single molecule FRET microscopy. In our assay, fluorescent donor and acceptor molecules were covalently attached to an RNA template mimicking the region of the HIV-1 genome in which the PBS sequence is located. We observe better folding of the RNA molecules in the presence of NC than with heat annealing and a large change in conformation of the RNA molecule upon tRNA annealing. Our results give further evidence that tRNA also interacts with the viral RNA PAS motif.

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Benchmark of a New Red-Enhanced Custom Technology Spad Detector for Single-Molecule FRET Experiments

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Single-molecule spectroscopy has relied on detectors that offer a variety of features enabling single molecule sensitivity. Single-Photon Avalanche Diodes (SPADs) are prominent among detectors used to sample fluorescent-labeled species and identify their physical characteristics, diffusion properties and elucidate molecular interactions. Existing brands of commercial SPADs suffer from either low quantum efficiency in one part of the spectrum, poor timing resolution or lack of temporal stability and/or heterogeneous instrument response functions (IRFs). Finally, some SPADs can fail after exposure to strong intensity signals. In this study, we characterize a new custom-technology SPAD detector addressing these drawbacks and compare its properties to those of a popular commercially available SPAD detector used in single-fluorescence microscopy experiments. We performed two-color single-molecule FRET (smFRET) experiments on doubly labeled DNA using Alternating Laser Excitation (ALEX). We compare results obtained with both types of detectors in identical conditions, using both ratiometric FRET and lifetime FRET analysis. The new detector offers excellent sensitivity in the red part of the spectrum. Lifetime FRET measurements reveal excellent timing resolution, IRF stability, enabling the study of short timescale dynamics. Our results validate this new red-enhanced SPAD detector for both intensity-based and time-resolved single-molecule FRET experiments among many other capabilities.

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Bayesian Inference Based FCS Analysis and Single Molecule Burst Analysis Reveal the Influence of Dye Selection on DNA Hairpin Dynamics

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Fluorescence-correlation-spectroscopy (FCS) is a powerful tool to gain information about dynamics of bio-molecules. However, the key problem is to extract the rates hidden in the FCS data by fitting the data to a meaningful model. A number of different fitting approaches have been described in recent years but the extraction of relevant information till now was still limited by numerous experimental problems and the fact that the set of starting parameter values chosen could often predefine the result. We establish a new way to globally analyze FCS data based on Bayesian inference to overcome these issues. Moreover, the influence of other remaining experimental error sources, e.g. photophysics, is excluded by additional means. Using this approach in combination with the results from single molecule burst analysis, we investigate the kinetics of DNA hairpins labeled with a variety of different fluorescent probes as a function of salt concentration. We find that dye selection influences the rates of hairpin opening and closing as well as the equilibrium constant of the transition. Thus, great caution has to be used when utilizing dye molecules as reporters for the kinetics of dynamic macromolecular structures.

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Probing Spatial Organization of mRNA in Bacterial Cells using 3D Super-Resolution Microscopy

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Bacterial cells are often erroneously regarded as a tiny sack of molecules, but growing evidence suggests surprisingly sophisticated internal organization in bacteria. Here we present how mRNA is spatially organized in a model bacterium, *Caulobacter crescentus*. We developed 3D super-resolution microscopy technique based on fluorescence in situ hybridization to probe chromosomally encoded mRNA of a specific gene with ~25 nm lateral resolution and ~50 nm axial resolution. We found that most of the mRNA molecules are localized near the gene loci on the chromosome and that the remaining mRNA molecules are distributed around cell area with interesting localization patterns in the axial direction. We investigated spatial distribution of mRNA in relation to the cellular morphology and discuss cell cycle-dependent localization of certain bacterial mRNA.