Single Molecule Visualization of the DEAH-Box ARPase Prp22 Interacting with the Spliceosome: A Dissertation

Eric G. Anderson

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SINGLE MOLECULE VISUALIZATION OF THE DEAH-BOX ATPASE PRP22 INTERACTING WITH THE SPLICEOSOME

A Dissertation Presented

By

Eric Gunnar Anderson

Submitted to the Faculty of the University of Massachusetts Graduate School of Biomedical Sciences, Worcester in partial fulfillment of the requirement for the degree of

DOCTOR OF PHILOSOPHY

JANUARY 5, 2016

BIOCHEMISTRY
SINGLE MOLECULE VISUALIZATION OF THE DEAH-BOX ATPASE PRP22
INTERACTING WITH THE SPLICEOSOME

A Dissertation Presented

By

Eric Gunnar Anderson

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JANUARY 5th, 2016
I would first like to thank Michael Egholm, my supervisor while I was a R&D scientist at Applied Biosystems working on peptide nucleic acids. Michael not only taught me how to be a successful industry scientist but pushed for my personal development goal of earning a PhD, even if it meant leaving his group. I hope to be able to repay the favor of mentoring young scientists as Michael did for me.

My project would not have been possible without the kind and patient assistance from Larry Friedman and Jeff Gelles. Thank you both for sharing in the wonders and tribulations of CoSMoS single-molecule experiments. I would also like to thank others involved in single-molecule splicing: Danny, Aaron and Inna for setting the bar, Andrew and Joerg for commiserating in the ways of methods development and Matlab image processing. To other members of the Moore lab, in particular Chris and Erin, for making the lab a great place to work and a difficult place to leave.

I will be always indebted to Melissa for my scientific training and advice on how to function on a mere four hours of sleep. During my First Year Retreat at Woods Hole, I was blown away by Melissa’s presentation, which included some of the first single-molecule splicing movies consisting of dim white spots flickering on a grainy black background. Those images of single spots took a hold of me then and have yet to quite let go. I am in awe of Melissa in the breadth and risk of her research interests and how she manages it all in an unassuming fashion. I will continue to look to Melissa as inspiration for scientific daring.

Finally I would like to thank my family, for without their support none of this would literally be possible. To my Mom and Pop for instilling in me the values of creativity, imagination and independence – critical traits for practicing science. It was not until starting a family of my own that I truly appreciated their efforts and sacrifices. To my in-laws, Debbie and John for raising a beautiful daughter who keeps me grounded and constantly showing the value of hard work. To Tyler and Oliver – I cannot describe the joy of being a father to you both and want nothing more than for you to freely explore what life has to offer you. I look forward to running all the miles, reading all the books with you and hearing the Tales of the Lion and the Fox continue with your children. And finally to Jillian, the love of my life and partner in all things. Without you I am nothing. I find myself forever happy with the family we have started together and look forward to realizing and enjoying every success with you.
In eukaryotes, the spliceosome is a macromolecular ribonucleoprotein machine that excises introns from pre-mRNAs through two sequential transesterification reactions. The chemistry and fidelity of pre-mRNA splicing are dependent upon a series of spliceosomal rearrangements, which are mediated by trans-acting splicing factors. One key class of these factors is the DEAH-box ATPase subfamily of proteins, whose members couple ATP hydrolysis to promote RNP structural rearrangements within the spliceosome. This is typified by Prp22, which promotes release of the spliced mRNA from the spliceosome and ensures fidelity of the second step of splicing. This role is well documented through classical biochemical and yeast genetics methods. Yet very little is known regarding the comings and goings of Prp22 relative to the spliceosome. My thesis research investigated the dynamics of Prp22 during splicing by using single-molecule fluorescence methods that allowed direct observation of these events. To do this, I helped construct a toolkit that combined yeast genetics, chemical biology and Colocalization Single Molecule Spectroscopy (CoSMoS) with in vitro splicing assays. Specifically, my thesis research consisted of CoSMoS splicing experiments in which fluorescently labeled pre-mRNA, spliceosome components and Prp22 were directly visualized and analyzed. Using these methods, I found that Prp22’s interactions with the spliceosome are highly dynamic and reversible. By simultaneously monitoring Prp22 and individual spliceosome subcomplexes, I was able to frame these Prp22 binding events in context relative to specific steps in spliceosome assembly and splicing. These experiments provide insight into how Prp22 promotes mRNA release from the spliceosome and maintains splicing fidelity.
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Some work reported in this dissertation has been published elsewhere.

Chapter 2 has been published previously as: (Anderson and Hoskins, 2014)
1.1 Splicing of precursor messenger RNAs by the spliceosome

The central dogma of gene expression consists of the transcribing information from genomic DNA into RNA copies that are translated into proteins. In eukaryotes, genes are in pieces separated by intervening non-coding regions called introns (Gilbert, 1978). Introns are not translated into proteins per se, but are important for regulation of gene expression (Moore and Proudfoot, 2009). The RNAs generated from the transcription of genomic DNA consist of the coding regions or exons, which are interrupted by introns. These transcripts are called precursor to messenger RNAs (pre-mRNAs) and are subject to a series of processing events within the nucleus resulting in the production and export of messenger RNA molecules (mRNAs) to be translated into proteins by the ribosome (Figure 1.1). Pre-mRNA splicing (referred herein as splicing) is one of the critical RNA processing events which consists of the removal of introns and joining of exons of an mRNA. Eukaryotic splicing is catalyzed by a multi-megadalton enzyme called the spliceosome (Brody and Abelson, 1985).
Figure 1.1: The central dogma in eukaryotes: A pre-mRNA is transcribed from genomic DNA and processed to an mRNA that is exported to the cytoplasm and translated by the ribosome into protein. Central to this processing is pre-mRNA splicing: the removal of introns and splicing of exons from a pre-mRNA.
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The spliceosome is one of many macromolecular machines involved in gene expression and its regulation. These machines often consist of RNAs and proteins interacting together, forming ribonucleoprotein complexes (RNPs). RNPs are intimately involved in and in some cases couple the processes of RNA transcription, splicing, transport/export, translation and silencing (Wahl et al., 2009). Indeed, the mRNA transcript itself is an RNP whose protein composition dramatically changes with each RNA processing event. These RNP machines are relatively large and can consist of hundreds of parts, which makes for challenging studies of their workings. Of these, the spliceosome is perhaps the most complicated and dynamic RNP, consisting of 5 small nuclear RNA-protein complexes called snRNPs (U1, U2, U4, U5 and U6), an all protein complex (the NTC or NineTeenComplex) and hundreds of proteins. Yet describing the spliceosome as vast machine belies its dynamic nature and invites comparison to another large and more stable RNP “-ome”: the ribosome. From a certain point of view, the spliceosome is a really a series of isolatable, assembly pathway intermediates. The question of why the spliceosome is so complex has been and continues to be intriguing and challenging to answer.

The unique complexity of the spliceosome can be addressed by examining its function as a cellular machine. Splicing plays a critical role in the broad range of evolutionary complexity of eukaryotes. Splicing in the budding yeast *Saccharomyces cerevisiae* (henceforth referred to as yeast) has been minimized to only 3% of its genes. These intron-containing genes have been
evolutionarily selected to serve essential roles in the life cycle of the yeast and require minimal mutation and regulated expression. On the other end of the range, the rich genetic diversity of higher eukaryotes is in large part due to the spliceosome-catalyzed splicing of pre-mRNAs from genes containing tens to hundreds of introns of vastly different lengths. By selecting different exon combinations, a single gene can encode many possible alternate mRNAs, which increases the functional repertoire of the cell. This process is called alternative splicing and is quite prevalent, with recent estimates of >95% (Pan et al., 2008).

Whether splicing a single intron in yeast or alternative splicing in humans, the core RNP parts of the spliceosome are highly conserved (Anderson and Hoskins, 2014). The conservation of spliceosome complexity is a tribute to its essential roles in maintaining the fidelity and regulation of gene expression.

1.2 The Chemistry of pre-mRNA Splicing

The spliceosome is the enzyme that mediates the removal of introns and joining of exons from pre-mRNAs. Introns vary considerably in length but contain the 3 sites used for the chemistry of splicing. Introns are flanked by conserved sequence elements consisting of 5’ and 3’ splice sites (SS) and contain a branch point sequence (BP). Figure 1.2A depicts a minimal pre-mRNA with the 5´SS, 3´SS and BP sequences from yeast. These yeast intron sequence elements are highly conserved, with 75% of introns having a GUAGGU 5´ SS, 95% with a
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branch site sequence of UACUAAC and 100% with a YAG 3' SS (Spingola et al., 1999).

The chemistry of splicing is straightforward and consists of 2 sequential 
Sn2-type transesterification reactions (Moore and Sharp, 1993) (Figure 1.2B).

---

**Figure 1.2**: Chemical mechanism of pre-mRNA splicing: A. pre-mRNA splicing requires identification of 5' and 3' splice sites and the branch site within the intron. Displayed are the yeast consensus sequences. B. Splicing occurs via two sequential transesterification reactions. Figure adapted from (Jurica and Moore, 2002).
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The first reaction consists of a nucleophilic attack of the phosphodiester bond at the 5′ splice site by the 2′ hydroxyl of the branch point adenosine. This results in a free 5′-exon and a lariat-exon intermediate consisting of the branched intron and 3′-exon. During the second step, the 3′ hydroxyl of the 5′ exon attacks the 3′ splice site, which joins the exons and liberates the lariat intron. Note that recent work by the Soo Chen-Cheng group has demonstrated the reversibility of the chemical steps of splicing (lariat formation and exon joining) and later showed competition between a novel debranching reaction and the reverse of the first step (Tseng and Cheng, 2008, 2013). These studies were done by changing the ionic environment (i.e., mono and divalent cations) within the catalytic core of the spliceosome.

The mechanism for self-splicing group II introns is quite similar to that of spliceosome catalyzed splicing (Valadkhan, 2010). This brings into question as to why did something as complex as the spliceosome evolve into an essential cellular machine? Given the minimal nature of information contained within the splice sites and branch point sequences, the problem is one of recognition of the correct sites for proper splicing. The spliceosome then is required for efficient and faithful splicing that is coupled to other processes of gene expression.

1.3 The Spliceosome Assembly Cycle

The prevailing view of the spliceosome is that it does not act as a preformed complex but rather assembles stepwise on the pre-mRNA substrate
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(Burge et al., 1999; Chen and Moore, 2014). The assembly cycle has been detailed in both yeast and mammalian systems using in vitro biochemical methods such as native gel electrophoresis, affinity chromatography and gradient centrifugation. The key stages of splicing have been operationally defined by the presence and absence of the snRNPs, the presence of reaction intermediates and products, and by native gel electrophoresis migration behavior (Will and Lührmann, 2011) (Figure 1.3). Under this model, the stages of assembly are precursors to the next one (Brow, 2002).

The first stage of assembly involves the recognition of the 5′ and 3′ splice sites and branch point sequence (BPS), resulting in E (early) complex. This initial step is ATP-independent, whereas all of the remaining stages are ATP-dependent. The U1 snRNP binds to the 5′ splice site while the branch point and pyrimidine tract are recognized by BBP (yeast) and U2AF complex (mammalian) respectively. The prespliceosome or A complex follows with BPS binding by the U2 snRNP, displacing BBP. The U4/U6*U5 tri-snRNP then binds to the 5′ splice site region, yielding B complex. At this stage, the spliceosome is catalytically inactive; extensive remodeling is required for the first step of splicing. This remodeling involves the binding of the NTC and loss of the U1 and U4 snRNPs, resulting in the activated spliceosome or B*complex. C complex arises after the first catalytic step, followed by more spliceosomal rearrangements from which the second step proceeds. Following completion of the second step, the spliced mRNA is released and the spliceosome is disassembled, allowing for reuse of its
Figure 1.3: Overview of the spliceosome assembly cycle. The spliceosome assembles step-wise on the pre-mRNA substrate forming a series of complexes. Emphasized is the dynamic nature of the spliceosome, with a branched pathway to A Complex, reversibility of all assembly steps and the splicing itself. After release of the spliced mRNA, the spliceosome is disassembled and its components available for another round of splicing.
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components for the splicing of other substrates. The assembly stages and associated rearrangements listed above exemplify the dynamic nature of the spliceosome and provide a glimpse into how splicing is regulated.

Although spliceosomal complexes are defined by their snRNP composition, proteins also play a critical role in splicing. By shear mass, proteins are thought to account for more than two-thirds of the spliceosome (Jurica and Moore, 2003). Mass spectrometry proteomics surveys have provided the parts list and highlighted the dynamic composition of the spliceosome (Fabrizio et al., 2009). Interestingly, the yeast spliceosome consists of ~90 proteins that are conserved within the ~300 spliceosomal proteins found in metazoans. As core components of the snRNPs, splicing proteins are intricately involved in providing the framework for splicing to occur and may even be involved in catalysis (Abelson, 2008).

1.4 DExD/H-Box Proteins Involved in Splicing

In addition to the snRNP and NTC proteins, there are other trans-acting proteins that act as splicing factors. This class of trans-factors is best typified by the SR proteins (serine/arginine-rich), which promote alternative splicing in metazoans and the DExH/D box proteins, whose actions as ATPases/helicases are required for remodeling of the spliceosome.

DExD/H-box proteins are pervasive in RNA metabolism events, ranging from transcription, splicing, transport and translation (Silverman et al., 2003).
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The DExD/H-box family is highly conserved and owe their name to the conserved sequence motif (Asp-Glu-Any-Asp/His). They are perhaps best known for their ability to unwind RNA duplexes in an ATP-dependent manner (Anna Marie Pyle, 2008). The helicase domain is responsible for the helicase and ATPase activity of these proteins. This ATPase activity is RNA-dependent and is thought to power translocation of the DEAH-box sub-group along their substrate. This helicase or unwindase activity has been demonstrated in vitro using generic nucleic acid duplexes and/or RNA-protein complexes (Jankowsky and Bowers, 2006).

DExH/D-box proteins are extensively involved in pre-mRNA splicing. These splicing trans-factors were first identified in a yeast genetic screen for splicing deficiencies (Lustig et al., 1986; Rosbash et al., 1981). Nearly all transitions and steps in splicing are governed by a DExH/D-box protein (Figure 1.4). Prp2, along with its binding partner, Spp2, are required for catalytic activation of the spliceosome by displacing the U2 snRNP subcomplex SF3b complex from the branch point region (Lardelli et al., 2010; Roy et al., 1995). Prp16 and Prp22 are respectively involved in the transition between the first and second steps, and the second step and mRNA release from the spliceosome. Finally Prp43 is required for lariat-intron removal and spliceosome disassembly after splicing has occurred (Martin et al., 2002). Not all splicing DExH/D-box proteins are external factors – the DEIH-box protein Brr2 is an integral part of the U5 snRNP and with the
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**Figure 1.4**: DEAH-box proteins drive splicing forward. The DEAH-box proteins Prp2, Prp16, Prp22 and Prp43 govern key steps in spliceosome activation, the splicing chemical reactions and disassembly. Prp2, Prp16, Prp22 and Prp43 are shown at the transitions where their actions are required. Also shown are the other trans-acting protein factors involved in splicing, as well as the known spliceosomal components and proteins released by the remodeling actions of the DEAH-box protein.
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GTPase Snu114 acts at temporally distinct stages during splicing (Maeder et al., 2009).

Prp16 is the most extensively studied of the spliceosomal DEAH-box proteins and was first identified in a yeast genetic screen for splicing deficient mutants (Couto et al., 1987). Prp16p is a DEAH-box ATPase that is capable of unwinding RNA duplexes \textit{in vitro} (Wang et al., 1998). While its helicase domain is highly conserved within the DEAH-box protein family, its unique N-terminal domain is required for binding to the spliceosome (Wang and Guthrie, 1998). Interestingly, when expressed as separate domains, the N-terminal and helicase domains can act together (i.e. \textit{trans}-complementation), recapitulating Prp16 functionality both \textit{in vivo} and \textit{in vitro}.

Prp16 acts during the transition between the first and second steps (Schwer and Guthrie, 1991). It is thought to use its ATPase activity to remodel the spliceosome conformation such that the substrate (free 5′-exon and lariat-intermediate) are positioned properly for the second transesterification reaction. How exactly it effects this change is not known, but recent evidence shows Prp16 facilitates the removal of the protein Cwc25, which binds near the branch point after activation (Lardelli et al., 2010; Ohrt et al., 2013; Tseng et al., 2011). As a \textit{trans}-factor, Prp16’s interaction with the spliceosome is transient and is no longer associated with the spliceosome prior to completion of the second step (Schwer and Guthrie, 1991).
Prp22 is a DEAH-box protein that acts during second step and promotes release of the spliced mRNA from the spliceosome (Company et al., 1991). Its function during the second step is ATP-independent and requires the ordered binding of the proteins Slu7 and Prp18 to the spliceosome (James et al., 2002). However, recent data from reconstitution and cross-correlation spectroscopy experiments from the Luhrmann lab shows only Slu7 and Prp18 but not Prp22 are required for the second step (Ohrt et al., 2013). Based on data from crosslinking and RNase H protection assays, Beate Schwer has proposed a model in which Prp22 facilitates mRNA release by binding downstream of the 3′-exon after exon ligation (Schwer, 2008). Prp22 then uses its ATPase activity to disrupt RNA-RNA and/or RNA-protein interactions between the mRNA and the spliceosome, perhaps the U5 components U5 snRNA-Loop I and Prp8 (Aronova et al., 2007).

The crystal structure of Prp43 provides some insight into how these DEAH-box proteins might work when modeled after the Ski2-like helicases (He et al., 2010; Walbott et al., 2010). Upon binding of the RNA substrate, the two recA domains form a pocket for ATP binding and hydrolysis. ATP hydrolysis is thought to power a ratcheting mechanism in which the duplex RNA substrate is pulled into and through a β-sheet, leading to strand unwinding and translocation of the helicase. Given the highly conserved nature of the DEAH-box proteins, it is tempting to ascribe a similar model to Prp2, Prp16, and Prp22.
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The DEAH-box proteins Prp2, Prp16, Prp22 and Prp43 also act as kinetic proofreaders in maintaining splicing fidelity (Figure 1.5). Kinetic proofreading was originally proposed as a model to account for fidelity of tRNA selection by the ribosome (Hopfield, 1974; Ninio, 1975). This model holds that optimal substrates are favorably selected through kinetic discrimination. That is, the selection process and catalysis are in competition with coupled ATP hydrolysis by in this case a DExD/H-box protein. If a suboptimal substrate is present, its catalytic rate will be slower than that of the ATPase, and will thus be discarded. In the context of splicing, Prp2 ensures proper activation of spliceosomes (Wlodaver and Staley, 2014), Prp5 proofreads U2 snRNA basepairing to the branch point region (Xu and Query, 2007), Prp16 maintains fidelity of BP recognition (Burgess and Guthrie, 1993; Koodathingal et al., 2010) while Prp22 proofreads the fidelity of exon joining (Mayas et al., 2006). Jon Staley’s group showed that Prp43 is not only involved in spliceosome disassembly post splicing, but also in a discard pathway when aberrant splicing is proofread by Prp16 or Prp22 (Mayas et al., 2010). This couples with work from the Soo Chen-Cheng lab that claimed Prp43 access spliceosomes after rearrangements mediated by Prp2, Prp16 and Prp22 (Chen et al., 2012). These observations are consistent with a model in which Prp43’s disassembly role is restricted to lariat intron spliceosomes if splicing is on pathway and fast relative to the activity of DEAH-box proteins. However, an off pathway splicing event with relatively slow kinetics
could be shuttled into a discard pathway by the DEAH-box protein governing that step.

Figure 1.5: Prp16 and Prp22 proofread the first and second steps of splicing. This scheme highlights the dual function Prp16 and Prp22 serve in splicing: their remodeling activities either promote splicing forward on pathway or a discard step. These pathways are determined by the kinetics of splicing versus the remodeling activity of Prp16 and Prp22.
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There remain several open questions concerning DEAH-box proteins and their roles as splicing proofreaders. The nature of the discard step for suboptimal substrates has not been fully addressed. Discard could entail repeated cycles of rejection and testing of substrates, or involve an irreversible spliceosome disassembly pathway. The kinetic details of the transient interactions of DEAH-box proteins with the spliceosome have not been determined. In particular, it is not known how their remodeling actions are timed or specified to their associated splicing events.

Given the dual roles as remodelers and proofreaders, each transition of splicing mediated by a DEAH-box protein is potentially a proofreading and/or remodeling event (see Figures 1.4 - 1.5). These questions of discard, timing, and specificity become more pertinent when the number of DEAH-box proteins involved in splicing is considered. Given the highly conserved nature of these proteins, one hypothesis is they share a common binding site within the spliceosome or are regulated commonly somehow at their correct time. This would imply that their binding is mutually exclusive, which has not been demonstrated. DEAH-box protein binding and therefore activity could be mediated by co-factors or even the conformation of the spliceosome itself (Smith et al., 2008). Another hypothesis is the conformational rearrangements made by one helicase provides the substrate for another, thereby forming a cascade of rearrangements (Cordin et al., 2012). Understanding the interactions of DEAH-
box proteins with the spliceosome and the molecular rearrangements that ensue will help provide a mechanistic picture of splicing.

Classical biochemical techniques have revealed a wealth of information concerning pre-mRNA splicing and spliceosome assembly. These techniques include \textit{in vitro} splicing assays, native gel electrophoresis, co-immunoprecipitation, cross-linking and nuclease protection assays. However, efforts to study the spliceosomal interactions of DEAH-box proteins using these biochemical tools have been hampered. These techniques often require purified spliceosomes or stalling of splicing at key intermediate states to provide synchrony. This stalling, be it from splicing inhibitors, mutant pre-mRNA substrates, or immunodepletion, is not always possible for all of the intermediate states.

Historically these limitations led to the use of unfractionated whole-cell extracts (WCE) from mammalian or yeast cultures for biochemical studies of splicing (Lin et al., 1985). WCE contain all of the components required for assaying splicing \textit{in vitro} and have provided the basis for the biochemical experiments used to elucidate the mechanisms involved in splicing. However, the splicing components in a WCE are subject to dilution effects from the loss of nuclear compartmentalization and possible degradation from nucleases and proteases. Until recently, the spliceosome has eluded the reach of classical biochemical techniques of using purified components to reconstitute splicing activity \textit{in vitro} (Warkocki et al., 2009). While an impressive feat, this is really a
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semi-reconstituted system in that B-complex spliceosomes are first prepared and arrested in a splicing assay with WCE. The arrested spliceosome can be forced along the forward pathway by adding the required trans-acting splicing proteins that are prepared recombinantly. The Lührmann group has used this method to investigate the disassembly pathway post-splicing and to dissect the second step (Fourmann et al., 2013; Ohrt et al., 2013).

1.5 Single-Molecule Approach

In order to gain a fundamental understanding of the mechanism of spliceosome-mediated splicing, new tools are required. Single-molecule methods first gained prevalence as a biophysical technique in the form of patch-clamp and single ion channel recordings during the 1970’s (Neher and Sakmann, 1976). Since then, single-molecule techniques have grown to include atomic force microscopy, optical tweezers and single-molecule fluorescence microscopy. Compared to ensemble-based studies, single-molecule studies provide very high sensitivity. This allows access to the underlying subpopulations and heterogeneity of complex systems. In terms of enzyme kinetics, single-molecule experiments can identify transient intermediates and multiple reaction pathways. These observations are often obscured by the averaging associated with ensemble methods.

Single-molecule studies have already provided a deeper understanding of the ribosome, motor proteins and RNA/protein folding (Blanchard et al., 2004;
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Nettels et al., 2007; Yildiz et al., 2003; Zhuang et al., 2000). What then, can single-molecule experiments tell us about splicing? Spliceosome catalyzed splicing is a highly complex and dynamic process involving many transitions and structural rearrangements. A single-molecule approach allows direct observation of splicing in real-time, including rare or transient interactions, without the need for synchronization. Single-molecule fluorescence (SMF) microscopy is particularly well-suited in this regard, and should be viewed as a complement to biochemical ensemble methods.

Pre-mRNA splicing is well suited for single molecule studies since the overall spliceosome assembly pathway and splicing reaction are known with an established *in vitro* assay. However, SMF splicing TIRF experiments require fluorescently labeled pre-mRNAs and proteins, for which new labeling methods had to be empirically developed. In particular, the model pre-mRNA had to be prepared piece-wise, site-specifically labeled with fluorophore(s), ligated together and derivatized with a biotin for attachment to the glass slide surface. In order to avoid dye photobleaching, an oxygen scavenging system compatible with splicing was developed. Early work by Aaron Hoskins and Danny Crawford demonstrated the feasibility of such an approach and for the first time visualized the ATP-dependent removal of a labeled intron (Crawford et al., 2008) (Figure 1.6).
Figure 1.6: Direct visualization of splicing using SMF microscopy. (a) Experimental scheme in which a pre-mRNA labeled with dyes is attached to the slide surface and able to report splicing via loss of intron fluorescence. (b-i) Time-lapsed TIRF images of wild-type and branch point mutant pre-mRNA showing loss of intron (yellow boxes). (j) SMF traces of wild type pre-mRNAs during splicing. (k) Plot of intron signal survival for wild type (diamond) and branch point mutant (X) pre-mRNAs during a SMF splicing reaction. Figure from (Crawford et al., 2008).
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Using a similar approach, a collaboration between the Christine Guthrie and Nils Walter labs used a dual-labeled pre-mRNA for observing splicing dynamics by single molecule FRET (smFRET) (Abelson et al., 2010a). By placing the FRET dye pair near the 5’ and 3’ splice sites, they found the pre-mRNA adopted highly dynamic conformations under in vitro splicing conditions (Abelson et al., 2010b) (Figure 1.7a). While intriguing, it is difficult to interpret the FRET dynamics without a reference to spliceosome assembly or splicing events. To resolve this issue they followed up with a study using a smFRET pre-mRNA in affinity-purified spliceosomes that were stalled before the activation step (Krishnan et al., 2013). By adding recombinant proteins Prp2 and Spp2 with ATP they were able to observe the pre-mRNA dynamics associated with spliceosome activation. Finally, they added fluorescently labeled Cwc25, a protein required for the first step, and observed FRET with the branch site presumably during the first step (Figure 1.7b).
Figure 1.7: smFRET reveals pre-mRNA conformational dynamics during splicing.  

**A.** Scheme for using a SMF-TIRF microscope for observing FRET between the splice sites of a pre-mRNA tethered to the slide surface. FRET is a measure of the dynamics of a pre-mRNA and can be monitored over time in different splicing conditions.  

**B.** Scheme for combining smFRET with labeled Cwc25 protein. After bleaching the acceptor dye on the pre-mRNA, FRET can be observed between the protein and the pre-mRNA during splicing. Figures adopted from (Abelson et al., 2010b; Krishnan et al., 2013).
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Pioneered by Jeff Gelles and Larry Friedman, the CoSMoS (Colocalization Single Molecule Spectroscopy) is a single molecule technique that uses TIRF microscopy to watch labeled biomolecules interact with a differently labeled molecule tethered to the slide surface (Friedman and Gelles, 2015; Friedman et al., 2006). The Gelles lab has used this technique to study the kinetics of transcription initiation (Friedman and Gelles, 2012).

Through a collaboration between the Moore and Gelles labs, CoSMoS has been used extensively to study splicing dynamics and spliceosome assembly. In order to observe splicing protein dynamics relative to pre-mRNAs tethered to the slide surface, specific protein labeling strategies had to be developed. These methods needed to be compatible with the whole cell extracts used in \textit{in vitro} splicing assays. After extensive trials and testing, we arrived at using an orthogonal combination of covalent (i.e. Snap-tag) and non-covalent (\textit{E. coli} DHFR-trimethoprim) protein fluorescent labeling technologies (Keppler et al., 2002; Miller et al., 2005) (see section 2.2.2).

Armed with labeled pre-RNAs and spliceosomal proteins, Aaron Hoskins first used the CoSMoS technique to directly observe spliceosome assembly events (Hoskins et al., 2011). He found spliceosome assembly in yeast is ordered and features reversible binding for all steps, with not one step being rate-limiting. Furthermore, all steps can lead to a discard pathway (Figure 1.8).
**Figure 1.8**: Dynamic assembly of the spliceosomes.  

A Preparation of fluorescent spliceosome complexes in whole cell yeast extracts for CoSMoS splicing experiments.  

B Single molecule traces of all spliceosome subcomplexes show reversible binding to a surface attached pre-mRNA.  

C Kinetic scheme of spliceosome assembly in which all assembly events are reversible and none are apparently rate limiting.  

Figures from (Hoskins et al., 2011)
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Inna Scherbakova followed this work by studying spliceosome assembly pathways for different yeast pre-mRNAs (Shcherbakova et al., 2013). She found a branched pathway where either U1 or U2 can bind first to make functional A-complex spliceosomes (Figure 1.9). It is particularly interesting to note the possibility of different spliceosome assembly pathways associated with different pre-mRNAs, since a majority of in vitro splicing uses one model pre-mRNA (i.e., ACT1 intron). This was also the first CoSMoS publication that introduced the use of a visual tool called rastergrams, which are able to convey the entirety of a single molecule experiment, in this case displaying the U1 and U2 binding events and the fate of the intron (see Figure 1.9D).

Finally, Danny Crawford combined CoSMoS with smFRET to pair pre-mRNA splicing dynamics with spliceosome assembly events (Crawford et al., 2013) (Figure 1.10A). Using a pre-mRNA labeled with a FRET dye pair at the branch point and 5′SS regions, he found that in buffer the pre-mRNA compacts, yielding a high FRET state. Once WCE was introduced, the branch point and 5′SS remain distal well into the spliceosome assembly cycle. By using WCE with labeled NTC complex, he observed a shift in the FRET state after arrival of the NTC complex and activation of the spliceosome (Figure 1.10B). This allows for more time for splice site selection later in the assembly pathway than previously appreciated.
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Figure 1.9 CoSMoS reveals a novel branched spliceosome assembly pathway.  
A  Scheme for CoSMoS experiment using labeled U1 and U2 extract and a pre-
mRNA with a labeled intron for monitoring splicing.  B  Intron signal loss is in 
WCE is ATP dependent.  C  Intron signal traces showing loss due to splicing 
(top) or photobleaching (bottom).  D  Rastergram showing U1 (red), U2 (green) 
or both (yellow) binding to a pre-mRNA with a labeled intron.  The rastergram is 
sorted by the time of intron loss (gray shading).  G  Model for U1 or U2 branched 
assembly pathway – both can lead to productive spliceosomes.  Figures from 
(Shcherbakova et al., 2013)
Figure 1.10: smFRET-CoSMoS shows pre-mRNA conformational dynamics relative to spliceosome assembly and activation. A. Scheme showing labeled pre-mRNA used for smFRET FRET state distribution of pre-mRNA in buffer. B. Heat maps of FRET states from a splicing experiment timed to arrival of labeled NTC. After NTC binding and delay of ~2 min, the pre-mRNA toggles between 2 FRET states. This continues until simultaneous departure of NTC and labeled intron. Figures from (Crawford, et al., 2013).
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This dissertation presents work previously published (Anderson and Hoskins, 2014) concerning the development of methods for constructing a CoSMoS splicing toolkit. These methods, particularly the development and optimization of fluorescent labeling of specific proteins in WCE, were critical for the CoSMoS splicing experiments detailed above. Finally, these methods were used to observe the dynamics of the Prp22 DEAH-box protein relative to spliceosome assembly and activation events and splicing itself.
Preface: The work presented in this chapter is reproduced from previously published work (Anderson and Hoskins, 2014), of which Aaron Hoskins and I are co-authors. I contributed by writing sections 2.2.1 and 2.2.2, preparing figures 2.1, 2.2, 2.3, 2.4, and 2.5, helping with the final proofing, formatting and editing. This work is reproduced with permission from the publisher (Springer, License # 3773310948905).
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2.1 Introduction

The application of single molecule techniques to analyze biochemical processes has become increasingly prevalent for elucidating complex reaction pathways and has been applied to a wide range of systems including replication, transcription, and translation (Dulin et al., 2013). These methods often measure fluorescence light emitted from a dye-labeled biomolecule [e.g., single molecule colocalization or fluorescence resonance energy transfer (FRET) experiments] or the response of a biomolecule to force (e.g., optical traps, magnetic tweezers, or atomic force microscopy) (Dulin et al., 2013). The ability to follow reaction trajectories of individual biomolecules is an extremely powerful approach for studying biochemical reactions, particularly when transient or low-abundance intermediates cannot be observed in bulk assays due to ensemble averaging (Weiss, 2000). Additionally, the elaborate assembly pathways for macromolecular machines can be easily deconvoluted using single molecule fluorescence colocalization assays to follow construction of a single complex from start to finish (Friedman and Gelles, 2012; Joo et al., 2013; Tsai et al., 2012).

Recently, single molecule approaches have begun to shed new light on the mechanisms of spliceosome assembly and the splicing of precursor mRNAs (pre-mRNAs) (Hoskins et al.) Unlike most single molecule experiments that are carried out using highly purified components, single molecule splicing reactions to date have been carried out in yeast whole cell extract (WCE). This has
presented a set of unique challenges at nearly each stage of the single molecule experiment—from fluorophore labeling in WCE to image acquisition to data analysis. Despite the experimental complexity, single molecule methods have been used to study both pre-mRNA conformational changes during splicing (Abelson et al., 2010a) and the dynamic interactions of spliceosome subcomplexes [the U1 and U2 small nuclear ribonucleoproteins (snRNPs), the U4/U6.U5 tri-snRNP, and the Prp19-associated complex (NTC)] with pre-mRNA (Hoskins et al., 2011). Both sets of experiments showed that a number of transitions along the splicing pathway appear readily reversible. Analysis of spliceosome assembly reaction kinetics further revealed that steps in this process are highly ordered on the RP51A substrate and no single step appeared to limit the rate of the overall assembly reaction (Hoskins et al., 2011).

The results described above concerning spliceosome assembly were obtained by CoSMoS—Colocalization Single Molecule Spectroscopy (Friedman et al., 2006; Hoskins et al., 2011). In CoSMoS experiments of splicing, the pre-mRNA is often attached to the surface and fluorescent spliceosome subcomplexes or splicing factors are monitored as they bind to and release from the pre-mRNA (Figure 2.1). These proteins or subcomplexes are labeled with different colors of fluorophores such that each species can be individually tracked and distinguished from the tethered pre-mRNAs.
Figure 2.1 Schematic overview of a CoSMoS experiment. (A) Drawing of a flow chamber with 4 lanes; the gray circle depicts an area imaged during an experiment (~400 µm²). (B) Magnified portion of a field of view from a 2-color CoSMoS experiment. The left square shows single molecules (spots) of pre-mRNA labeled with a red fluorophore and fluorescence imaged at > 635 nm. The right square shows the same field of view with single molecules of a SNAP-labeled spliceosome protein bound to the surface-tethered pre-mRNA and imaged with a green fluorophore at < 635 nm. (C) Drawing of a SNAP-labeled Prp16p molecule interacting with a surface tethered pre-mRNA. The pre-mRNA is attached to the slide through a biotin:streptavidin:biotin linkage.
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CoSMoS experiments are enabled by both surface tethering of the pre-mRNA and TIRF illumination. The evanescent wave of energy that is used to excite fluorophores by TIRF dissipates rapidly from the glass/water interface. This means that only fluorophores within ~100nm of the surface become excited and emit light. Additionally, molecules must remain fixed in position for a time period comparable with the camera frame rate to be observed in a CoSMoS experiment, thus, necessitating surface tethering of one of the fluorescent components for viewing discrete “spots” of single molecule fluorescence. Molecules that transiently pass through the evanescent field cannot be discerned as discrete “spots” but blur into the background since the camera frame rate in most microscopy experiments (maximum speed of ~500 fr/s) is much slower than diffusion. Consequently, experiments can be performed with free, fluorescent molecules in solution at concentrations <100 nM. The surface tethering is accomplished either by direct attachment of a biomolecule to a surface or indirectly by binding interactions between a fluorescent biomolecule in solution and its immobilized partner. It is critical that the surface be sparsely populated with biomolecules to ensure that fluorescent spots of single molecules are being separately observed.

Key to the implementation of a multi-color CoSMoS experiment is the efficient detection of photons emitted from different fluorophores excited by lasers of different wavelengths. This can be accomplished using a TIRF microscope design pioneered by the Gelles laboratory called micromirror TIRF.
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(mmTIRF) (Friedman et al., 2006). In mmTIRF, small broadband mirrors (mm in size) are used to direct the excitation laser light into and out of the microscope objective. This leaves the center of the objective free for fluorescence emission and unobstructed by dichroic mirrors found in other designs. Details on the construction of a mmTIRF microscope for CoSMoS as well as other TIRF microscope configurations have been published elsewhere (Friedman et al., 2006; Selvin, 2008).

In this chapter, we focus on the preparation of three components of a typical CoSMoS splicing assay: synthesis of a fluorescent and biotinylated pre-mRNA, labeling of spliceosome proteins in a yeast whole cell extract (WCE) with the SNAP tag, and assembly of flow chambers for an objective-based TIRF microscope. We then provide a protocol for putting these components together to perform a CoSMoS experiment between a surface tethered pre-mRNA and a single, SNAP-labeled spliceosome component. Due to the diversity in microscope designs and software implementation, we do not focus in this chapter on the specifics of image acquisition and processing, as this will vary lab-to-lab.

2.2 Materials

2.2.1 Design of Fluorescently-labeled pre-mRNA Substrates

In order to monitor spliceosome assembly and/or RNA splicing by CoSMoS, pre-mRNA substrates are immobilized on a streptavidin-coated glass
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surface and their locations determined by fluorescence. We typically construct two types of fluorescent pre-mRNAs for CoSMoS: location reporters and splicing reporters. Location reporters contain a single fluorophore and biotin located near the 3’ end of the pre-mRNA. Splicing reporters contain fluorophores located in either the 5’ or 3’ exon or intron in addition to a biotin modification at the 3’ end. The construction of splicing reporters has previously been described in detail (Abelson et al., 2010b; Crawford et al., 2008, 2013) and is beyond the scope of this article. We will instead focus on the more straightforward construction of location reporters. We have found that the most versatile approach is to incorporate a fluorophore and biotin into a short oligonucleotide (oligo) or “handle” that is ligated to the pre-mRNA 3’ end in a single step rather than direct modification of the RNA transcript. This approach allows a great deal of flexibility in the choice of fluorophore and pre-mRNA substrate.

Similar to ensemble experiments, relatively few pre-mRNA substrates have been used in single-molecule studies. For studying the yeast spliceosome, we often use the RP51A, UBC4, or ACT1 pre-mRNAs, all of which splice well in vitro. The pre-mRNA is prepared by transcription with T7 RNA polymerase using a PCR-generated template (see Note 1). The pre-mRNA can be capped during transcription by addition of cap analog dinucleotide or after transcription using an enzymatic capping system. The transcript may also be trace-labeled with radioactive $\alpha$-[32P]-UTP. This facilitates accurate quantification of the pre-mRNA and eliminates the need for exposing the RNA to potentially damaging UV
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radiation (Greenfeld et al., 2011). The levels of radiation used are miniscule and often undetectable with a Geiger counter in a single molecule assay. Methods for preparation of pre-mRNA substrates by transcription have been published elsewhere (Crawford et al., 2008, 2013; Moore and Query, 2000).

For the “handle” that will be ligated to the 3' end of transcript, we use a commercially prepared 27-nucleotide oligonucleotide containing ribose 2'-O-methyl modifications to prevent degradation and triggering of RNaseH cleavage during the splicing assay. The biotin is incorporated during synthesis at the 3' end, and the oligo includes a 5-(2-aminoallyl)uridine to facilitate labeling with N-hydroxysuccinimide (NHS) activated fluorophores. These oligos can be purchased from a number of commercial suppliers such as IDT or Dharmacon/Thermo Scientific. Conditions for labeling the oligo with fluorophores and purification of the labeled oligo have been previously described (Crawford et al., 2008).

The fluorescent handle oligo is 5' phosphorylated using polynucleotide kinase (PNK) and joined to the pre-mRNA via splinted ligation with RNA ligase. Either T4 RNA Ligase 1 or 2 (RNL1 or 2) can be used, though we often use RNL1 and a protocol developed by Stark et al. for this particular junction (Stark et al., 2006). Since RNL1 is a single-stranded ligase, the splint is designed such that the 3' and 5' ends of the RNA and biotin handle, respectively, are free but in close proximity (Figure 2.2). If RNL2 is used, then the splint is designed to directly abut the two ends being joined (Figure 2.2). Protocols for using RNL1
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and RNL2 are similar and may need to be optimized for each junction by adjusting the ligation time, temperature, amount of enzyme, or ratios of the RNA fragments and splint oligo.
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Figure 2.2 Schemes for splinted ligation to prepare fluorescent, biotinylated pre-mRNAs. The bold P and OH indicate the 5' phosphate 3' hydroxyl groups of the donor biotin handle and acceptor pre-mRNA, respectively. Sequences shown are for the RP51A pre-mRNA substrate. The biotin handle contains 2' O-methyl residues (lowercase m), a fluorophore (star) attached to a 5-aminoallyl-uridine (5-N-U), and a 3' biotin (Bio). The DNA splints are continuous, but here represented as two distinct portions separated by lines. **(A)** Splint and junction design for ligation with RNA ligase 2 (RNL2), a double stranded RNA ligase. **(B)** Splicing and junction design for ligation with RNA ligase 1 (RNL1), a single stranded RNA ligase. Figure adapted from reference (Stark et al., 2006).
2.2.1.1 Ligation of a fluorescent biotin handle to a pre-mRNA

1. \([^{32}\text{P}]\)-labeled pre-mRNA transcript, gel purified (1 equivalent, 28 pmol in \(\leq 8 \mu\text{L of H}_2\text{O}\))
2. Fluorescent biotin “handle” oligonucleotide (2 equivalents, 56 pmol in \(\leq 3 \mu\text{L of H}_2\text{O}\))
3. DNA splint oligonucleotide (1.5 equivalents, 39 pmol in \(\leq 2 \mu\text{L of H}_2\text{O}\))
4. T4 Polynucleotide Kinase and 10x PNK Buffer
5. ATP (700 \(\mu\text{M and 20 mM stocks in H}_2\text{O, each prepared fresh from an aliquot of a 100 mM stock solution at pH \sim 7}\))
6. RNasin Plus RNase Inhibitor (optional)
7. T4 RNA Ligase 1 (RNL1) and 10x RNL1 Buffer
8. RNase free deionized H\(_2\)O, not DEPC treated
9. 1x TBE: 8.9 mM Tris-base, 8.9 mM boric acid, 2 mM EDTA
10. Denaturing polyacrylamide gel: 6%, acrylamide:bis 19:1, 8M urea, 1x TBE, dimensions \(~20\times26\) cm with \(~0.8\) mm thick spacers
11. Gel loading buffer: 90% deionized formamide, 1 mM EDTA, 0.03% w/v bromophenol blue, 0.03% w/v xylene cyanol
2.2.2 Generation of Yeast Extracts Containing Tagged Proteins

CoSMoS splicing experiments often require that spliceosome proteins be fluorescently labeled. This represents a considerable challenge, particularly if the experiments are to be conducted in a WCE. While it is possible to add recombinant proteins back to a WCE, many spliceosome proteins are difficult to work with and these experiments could necessitate prior depletion of the endogenous protein to avoid competition between the native and fluorescent molecules. As an alternative approach, chemical tools can be combined with yeast genetics to modify spliceosome proteins with N- or C-terminal protein tags.
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*in vivo* by homologous recombination (Burke et al., 2005). Small molecule fluorophores can then be added to a WCE containing the tagged proteins to obtain fluorescent spliceosome components.

Tagging endogenous proteins by homologous recombination offers several advantages. First, if the yeast strains employed are haploid and proteins essential for viability are tagged, then survival of the yeast strains is a good indicator that the tagged proteins are functional *in vivo*. The effect of the tag on yeast growth can also be monitored and compared to the parental strain, as can the *in vitro* splicing activity of the WCE. Often, but not always, we have found that strains which grow poorly also produce WCE with poor splicing activity. Another advantage of tagging endogenous proteins is that it eliminates complicated procedures involving expression, purification, and labeling of recombinant proteins. The specific activity of the recombinant protein may be difficult to determine, and high concentrations are often needed to restore splicing activity in depleted extracts. These concentrations may exceed the limit imposed by the TIRF measurement (<100 nM fluorophore in solution).

The limit on solution fluorophore concentration also impacts which proteins can be tagged in the WCE by homologous recombination and subsequently visualized by CoSMoS. We have found that many spliceosome proteins are likely present in concentrations of <10 nM in a WCE and many can easily be tagged and visualized by CoSMoS. Other proteins (such as those involved in translation) are present at much higher concentrations and these
fluorophore labeled proteins could increase the background in the experiment to the point that the surface can no longer be discerned. The yeast GFP database (Huh et al., 2003) is an excellent resource for determining the suitability of a protein target for tagging and subsequent CoSMoS experiments. If a highly abundant WCE protein is being studied, there are several single molecule technologies that can make these experiments possible (Leslie et al., 2010; Loveland et al., 2012; Uemura et al., 2010).

While there is a plethora of protein labeling technologies available, aspects of both in vitro splicing and the CoSMoS experiment itself place constraints on the applicable methods. Since proteins will be labeled in WCE, labeling must be highly specific for the protein of interest. If proteins are present at low levels in the WCE, the labeling method must be sufficiently rapid to ensure a high degree of protein derivatization (ideally quantitative) under conditions that will retain the splicing activity of the extract (~30 min at room temperature or ~3h at 4°C). Finally, CoSMoS experiments are greatly facilitated by the bright and stable fluorescence signals observed from organic fluorophores. Many fluorescent proteins either photobleach too rapidly for studies of splicing lasting tens of minutes, are too dim to observe easily as single molecules, or display unfavorable photophysical properties (i.e., blinking) that can confound analysis.

With these constraints in mind, we empirically determined that two component systems relying on a protein tag and small molecule ligand were the best suited for labeling spliceosome proteins in WCE. In our laboratories, we
have often used either the *E. coli* dihydrofolate reductase (EcDHFR) tag developed by the Cornish laboratory (marketed by Active Motif) (Miller et al., 2005), the SNAP or CLIP tags developed by Johnsson and coworkers (marketed by New England Biolabs) (Keppler et al., 2002), or the Halo tag developed and marketed by Promega (Ohana et al., 2009). For the purposes of this chapter, we will focus on the SNAP tag.

SNAP tag labeling utilizes a modified human DNA repair enzyme (\(\text{O}^6\)-alkylguanyl-S-transferase) that becomes alkylated at an active site thiolate in the presence of \(\text{O}^6\)-benzylguanine (bG) derivatives (**Figure 2.3**). We have found that for the SNAP tag protein and most bG fluorophores efficient labeling occurs with 2 \(\mu\)M bG fluorophore in 30 min at 20\(^{\circ}\)C in WCE. However, this rate can vary for different dye substrates and needs to be determined experimentally for each dye/tag pair. Recently a variant SNAP tag with improved reaction kinetics has been reported ("fast SNAP" or SNAP\(_f\)) (Sun et al., 2011). With the SNAP\(_f\) tag, efficient labeling can often be achieved with 500 nM dye in \(~15\) min at 20\(^{\circ}\)C. In addition to the SNAP tag, the CLIP tag has also been developed along with its "fast CLIP" counterpart, CLIP\(_f\) (Gautier et al., 2008). Rather than being reactive towards bG derivatives, the CLIP tags react with benzylcytosine (bC). We have used the CLIP tags successfully for labeling spliceosome proteins albeit with slower kinetics and higher substrate requirements than for the SNAP tags.

A variety of fluorophore bG derivatives are available from New England Biolabs or can be synthesized easily from bG-amine building blocks. In practice,
we have found that unpredictable interactions of the fluorophores with the yeast extract, stickiness of the fluorophore to glass surfaces used during microscopy, or unwanted photophysical properties (e.g., blinking) have limited the choice of fluorophores that can be practically used in a CoSMoS experiment. In general, the bG derivatives of Atto-488, DY549, and DY647 (sold as SNAP-Surface ® 488, 549, and 647, respectively, by New England Biolabs) work well in WCE and for CoSMoS experiments.

During SNAP labeling in WCE, the bG fluorophore is in excess over the spliceosome protein being labeled. The free dye must then be removed prior to the CoSMoS experiment. We have concluded that dialysis is completely ineffective for dye removal from labeled extracts, therefore size-exclusion chromatography (SEC) is used. We developed a SEC method in which efficient dye removal was balanced against maintaining splicing activity by minimizing extract dilution. This SEC method is technically a group separation or desalting step, with the extract collected in the column void volume while the free dye remains in the column. With practice, this method can reduce free bG fluorophore background to <10 nM while maintaining the splicing activity of the WCE.
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Figure 2.3 Reaction scheme for SNAP-tag protein labeling. The SNAP-tag protein reacts with an $O^6$-benzylguanine dye substrate, transferring the fluorophore to an active site cysteine.
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2.2.2.1 Labeling of Spliceosome Proteins with Fluorophores in Whole Cell Extract

1. Yeast whole cell extract (WCE) from a SNAP-tagged strain (1.2 mL aliquot, see Note 2)
2. SNAP-tag bG dye substrate (~1mM in DMSO, New England Biolabs, see Note 3)
3. SEC Buffer: 25mM HEPES-KOH pH 7.9, 50mM KCl, 1 mM DTT, 10% v/v glycerol
4. Sephadex G-25, 50% slurry in water
5. Low pressure liquid chromatography (LPLC) column (e.g., Kontes Flex Column, 0.7 x 15cm)
6. LPLC luer-lock fittings (2x 3-way stopcocks, 1x barbed adapters for pump tubing)
7. Peristaltic pump (e.g., Pump P-1, GE Lifesciences)
8. Liquid N₂ and dewar

2.2.3 Design of Flow Chambers for TIRF Microscopy and Single Molecule Experiments

Like many other microscopy techniques, the single molecule CoSMoS splicing assay is carried out on glass slides. These assays can be conducted on TIRF microscopes employing either a prism-based or objective-based illumination scheme. With prism-based illumination, the excitation source is directed onto the sample via a prism positioned on top of the glass slide.
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When an objective-based illumination scheme is employed, the excitation laser is directed onto the sample from the bottom of the glass slide. Due to these differences in geometry, different styles of flow chambers must be used for each illumination scheme. The preparation of flow chambers for prism-based TIRF microscopy has been described elsewhere (Selvin, 2008; Zhao and Rueda, 2009). Here we illustrate how to manufacture simple flow chambers for an objective-TIRF microscope.

For colocalization experiments, we manufacture flow chambers using disposable glass microscope slides. It is critical that the glass slide closest to the objective be of the proper thickness (typically No. 1.5 cover glass which is ~0.17mm thick) to obtain images of high quality. For experiments in which FRET is also monitored, then the slide should be made from quartz or fused silica to eliminate the high background signal due to impurities found in lower grades of glass. Fused silica slides are extremely fragile, expensive, and non-disposable. Instructions for the preparation of No. 0 fused silica slides for CoSMoS can be found in other resources (Crawford et al., 2013). Note that these fused silica slides are both thinner and possess a different refractive index than No. 1.5 cover glass; consequently, adjustments in the TIR angle and microscope optics are necessary to obtain high quality images. Fused silica slides and coverslips can be purchased from suppliers such as SPI Supplies.

Whether glass or fused silica slides are used in the experiment, the slide and coverslip must be scrupulously cleaned before use. We often employ a
sonic water bath for this purpose. Alternatively, a plasma cleaner can be used if one is available (Selvin, 2008). It is important that the cleaned glass is protected from airborne dust particles, and in some environments the slides may need to be cleaned and assembled under HEPA air filtration such as a PCR workstation or in a hood with horizontal laminar flow (AirClean Systems). As a part of the cleaning procedure, the glass is activated for silanization and derivatization with amine reactive polyethylene glycol (PEG) reagents. Adequate passivation of the slide with PEG or other molecules is essential for single molecule experiments to prevent non-specific binding of biomolecules to the glass surface. In addition, biotin can be incorporated onto the slide surface at this stage for biomolecule attachment via PEG-biotin conjugates and streptavidin. While the methods described below have been found to facilitate a great number of experiments, occasionally passivation protocols must be optimized by varying the surface chemistry, addition of BSA or nucleic acids, or altering the PEG molecule length in order to obtain sufficient passivation (Alemán et al., 2009; Revyakin et al., 2012) (28, 29). Once assembled and passivated, the slides can be stored at 4°C for up to one week. We find that the lowest amount of non-specific binding typically occurs on slides < 24 hours old, and the degree of non-specific binding increases with slide age. In some cases the derivatized slides can be washed, dried, and stored at -80°C with desiccant for longer periods of time. These slides are often adequate for day-to-day experiments but may possess a higher degree of non-specific surface binding compared to freshly prepared slides.
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For many experiments, flow chambers can be constructed from simply two pieces of glass separated by a thin layer of vacuum grease as described below. In this configuration, capillary action is used to introduce liquid into each chamber and liquids are wicked out using filter paper. These slides typically have 4 chambers, each with a volume of ~20 µL. More complicated flow chambers with altered geometries, attachment points for inlet and outlet tubing, or that use alternative materials to vacuum grease [e.g., polydimethylsiloxane (PDMS)] can also be constructed. However, we find that the chamber configuration described below is often suitable for a wide-range of CoSMoS experiments.

After the flow chambers and fluorescent RNAs and extracts have been prepared, the CoSMoS experiment is ready to be conducted. The exact protocol will depend on the configuration of the microscope and flow chamber as well as the nature of the experiment. In this chapter, we describe only the fundamentals of surface attachment of a fluorescent RNA and introduction of the WCE. It should be noted that these experiments often require the addition of an enzymatic oxygen scavenging system and triplet state quenchers to extend fluorophore lifetime and limit blinking (Aitken et al., 2008; Crawford et al., 2008; Rasnik et al., 2006; Swoboda et al., 2012) (see Note 4).

The image acquisition protocol, microscope controls, and data processing routines will vary depending on the software and hardware preferences of each laboratory. While many laboratories utilize custom software to analyze single molecule data, several computer programs have recently become available to
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facilitate this stage of the experiment (Bronson et al., 2009; Dave et al., 2009; 
Greenfeld et al., 2012; McKinney et al., 2006; Milescu et al.). The nuances of 
interpreting single molecule data have been described in detail in many 
publications and great care must be taken to account for the observation of a 
single molecule event as well as the probability of having seen that particular 
event during the experiment (Neher and Sakmann, 1976; Schnitzer and Block, 
1995).

2.2.3.1 Preparation of Flow Chambers for Objective TIRF Microscopy

1. Micro-90 Cleaning Solution (M-9050-12, International Products 
   Corp.)
2. KOH (100 mM solution in MilliQ H$_2$O)
3. Ethanol (200 proof)
4. MilliQ H$_2$O
5. Vectabond (SP-1800, Vector Laboratories) (see Note 5)
6. Acetone (spectrophotometric grade, 99+%) 
7. Sodium bicarbonate
8. Vacuum grease (e.g., Dow Corning high vacuum grease)
9. Compressed nitrogen gas (ultra high purity)
10. Biotin-PEG (biotin-PEG-SVA MW 5000, Laysan Bio) (see Note 
    5)
11. PEG succinimidyl valerate MW 5000 (MPEG-SVA-5000, 
    Laysan Bio) (see Note 5)
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12. clear nail polish (optional)
13. 25 x 25mm cover glass (Corning No. 1.0 or 1.5)
14. 24 x 60mm cover glass (Gold Seal No 1.5, No. 3423)
15. Plastic disposable luer lock syringes (3-10 mL)
16. Empty pipette tip boxes with inserts (e.g., TipOne boxes, USA Scientific)
17. Razor blades or metal slide holders
18. Sonic water bath (e.g., VWR Symphony)
19. Slide Mailers x 5 (Fisher Scientific)
20. Cover glass forceps
21. Wafergard GN Gas Filter Gun (Entegris)
22. Syringe filters (0.20 µm, regenerated cellulose)

2.7 Assembling a Single Molecule Splicing Experiment

1. Biotinylated, fluorescent pre-mRNA
2. SNAP labeled yeast WCE
3. Cleaned and passivated glass slide
4. Dithiothreitol (DTT, 1 M stock solution, 0.20 µm filtered, aliquoted and stored at -20°C)
5. PEG 8000 (15% w/v solution in MilliQ H₂O, 0.20 µm filtered)
6. Potassium Phosphate (500 mM in MilliQ H₂O, pH 7.3, 0.20 µm filtered)
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7. 3,4-dihydroxybenzoic acid (PCA, 50 mM in MilliQ H₂O, aliquoted and stored at -80°C, the PCA may be recrystallized from hot MilliQ H₂O to increase solubility)

8. Protocatechuate 3,4-dioxygenase from *Pseudomonas* (PCD, resuspended to a concentration of 24-48 U/mL in 50 mM Tris base pH 8.0, aliquoted and stored at -80°C)

9. (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (trolox)

10. 200x triplet quencher master mix in DMSO (optional): 100 mM propyl gallate, 200 mM 4-nitrobenzyl alcohol, 200 mM cyclooctatetraene, aliquoted and stored at -80°C

11. Streptavidin (10 mg/mL aliquots in PBS, stored at -80°C, Prozyme, SA10)

12. Nuclease Free Bovine Serum Albumin (100 mg/mL)

13. ATP (100 mM, pH 7, aliquoted and stored at -80°C, optional)

14. MgCl₂ (1 M stock solution, 0.20 µm filtered)

15. RNasin Plus RNase Inhibitor (Promega, optional)

16. Filter paper (Whatman 1, 90 mm)

17. Plastic disposable luer lock syringes

18. Syringe Filters (0.20 µm, regenerated cellulose)

19. Low adhesion pipette tips and microcentrifuge tubes
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2.3 Methods

2.3.1 Ligation of a fluorescent biotin handle to a pre-mRNA transcript

1. In a small PCR tube, combine the fluorescently labeled biotin “handle” oligo (56 pmol) with 0.5 µL of 10x PNK buffer, 0.5 µL of 700 µM ATP, 0.5 µL of T4 PNK (5 U), and RNase Free H2O for a final volume of 5 µL. Avoid prolonged exposure of fluorescent materials to light.

2. Incubate in a PCR machine at 37°C for 60 min to phosphorylate the biotin “handle”. Heat inactivate PNK by incubation for 20 min at 65°C. Spin down the PCR tube to collect liquids in the bottom.

3. In a separate PCR tube, combine the pre-mRNA transcript (28 pmol), the DNA splint oligo (39 pmol), the phosphorylated biotin “handle” (56 pmol), and RNase Free H2O to a final volume of 14-15 µL.

4. Anneal the pre-mRNA and biotin handle to the splint by incubating at 65°C for 3 min in a PCR machine followed by a room temperature incubation for 5 min.

5. Add 1 µL RNasin, 2 µL 10 X RNL1 Buffer, 1 µL of 20 mM ATP, and 1-2 µL RNL1 (10-20 U) to a final volume of 20 µL.

6. Incubate at 37°C for 60 min in a PCR machine.
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7. While the ligation reaction is incubating, pre-run a 6% acrylamide denaturing gel for at least 30 min to an operating temperature of ~50°C. It is best to run the gel either in a darkened room or inside a large cardboard box to protect the ligated fluorescent RNA from light.

8. Once the ligation reaction has been completed, add an equivalent volume of dye-free loading buffer (20 µL) to the reaction and load onto the pre-run gel. Include loading buffer containing bromophenol blue and xylene cyanol in an adjacent lane in order to track the progress of the electrophoresis.

9. Carry out gel electrophoresis at constant power (35W) for 1.5-2.5 h, depending on the size of the RNA transcript and the acrylamide gel being used.

10. Dismantle the gel from the electrophoresis apparatus and remove one of the glass plates. Cover the gel and remaining glass plate with plastic wrap. To locate the ligation product, cut small shapes (3-4) out of Whatman filter paper and soak each in a radioactive solution of similar activity to the trace-labeled RNA. Using clear tape, adhere the filter papers to the plastic wrap in an asymmetric pattern around the lane that contains the ligation product. Expose the gel to either X-ray film (~1-5 min) or a phosphorimager screen (~1-2 min). From the developed X-ray film
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or phosphorimage, create a 1:1 replica copy of the gel on paper and cut out the locations of the filter paper shapes and the RNA ligation product (usually the upper of two RNA bands) (Figure 2.4).

Figure 2.4 Denaturing polyacrylamide gel purification of the pre-mRNA-biotin handle following ligation. The ligation product (upper band) is well separated from the unmodified transcript (lower band). The dark polygon on the right originated from a radioactive marker used to determine the location of the ligation product before it was excised from the gel. (Gel courtesy of Joshua Larson, U. Wisconsin-Madison.)
11. Using the paper template, excise the ligated pre-mRNA product and place into a 1.5 mL microcentrifuge tube. It is often beneficial to cut the RNA band into small pieces (~1 mm²) and to avoid including any excess acrylamide. Add 400 µL of gel elution buffer to the tube and centrifuge or vortex briefly to immerse the gel slice in the buffer. Freeze the gel slice with dry ice (~5-10 min), wrap the tube with aluminum foil to protect the RNA from light, and rotate the tube end-over-end for ~16h at room temperature.

13. After incubation, briefly centrifuge the tube to pellet the acrylamide gel slices (12,000 rpm, ~1 min). Transfer the extracted RNA (supernatant) to a new tube and add 1.2 mL ice cold EtOH.

14. Incubate at -80°C for ≥ 1 h, then centrifuge at 12-14,000 rpm for 30 min at 4°C. Remove the supernatant and wash the pellet with 70% EtOH and centrifuge as before for 5 min.

15. Remove the supernatant and air-dry the pellet for 5 min at room temperature.

16. Resuspend the pellet in 50 µL nuclease-free water. Store the RNA in ~10 µL aliquots at -80°C. Protect the RNA from light using amber tubes for storage and/or a light proof container.

17. The pre-mRNA concentration can be determined by scintillation counting using a sample of known concentration as the reference.
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(often a sample of the trace-labeled transcript before ligation or an aliquot of the transcription reaction used to produce the RNA).

2.3.2 Labeling of SNAP Proteins in WCE

1. Assemble a low pressure chromatography column with a stopcock and attach to a ring stand in a 4°C cold room (Figure 2.5A). With the stopcock closed add 3.8 mL of water and mark the height of the water on the side of the column as a reference line. Add 0.2 mL of additional water and mark a second reference line (4.0 mL). Drain the water from the column.

2. Add 7-8 mL of a ~50% Sephadex G25 suspension to the column. Open the stopcock and allow the column to begin to drain by gravity. Do not let the column run dry and avoid cracks or channels in the resin.

3. Set up and prime a peristaltic pump with SEC buffer, turn off the pump, connect the tubing to the inlet at the top of the column, and turn the pump back on to maintain a flow rate of ~0.4 mL/min once the resin has packed.

4. Allow the column bed to compact. If necessary, adjust the bed height to a position between the 3.8 and 4 mL reference marks. This can be done by using a glass pipet or tuberculin syringe to
resuspend the upper portion of the resin bed and adding or
removing resin as needed.

5. Equilibrate the column in SEC buffer for at least 2 hr.

6. Thaw a 1.2 mL aliquot of yeast extract containing SNAP-tagged
proteins on ice.

7. Add SNAP-tag bG fluorophore substrate to a final concentration
of 1 µM for the SNAPtag or 2 µM for the SNAP tag. Mix the solution well by inversion.

8. Incubate in the dark for 30 min at room temperature, mixing
every 10 min. After 30 min, place the extract on ice and immediately proceed to the column purification.

9. Stop the peristaltic pump. Using a glass pipet or tuberculin syringe, gently remove the buffer above the column bed, taking care not to disturb the resin. Allow any remaining buffer on top of the resin to drain from the column. Once all of the buffer has entered the resin, close the stopcock to prevent the column from drying.

10. Slowly and gently add the labeled yeast extract to the column directly on top of the resin bed being careful to disturb it.

11. Open the stopcock and allow the extract to drain into the resin.
Use a 10 mL syringe fitted with a 3-way stopcock to apply manual pressure to the top of the column to increase the flow rate to ~0.5
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mL/min (Figure 2.5B). The resin will pack (shrink) during application of the extract. After ~1 mL of extract has entered the resin, begin collecting the column eluate in 1.5 mL microcentrifuge tubes (0.4-0.5 mL per fraction).

12. Once the extract has entirely entered the resin, release the pressure, close the stopcock at the base of the column, and remove the syringe and column top. Carefully add SEC buffer on top of the resin and continue adding SEC buffer till the column has nearly filled (~4-5 mL). Re-attach the column top and reconnect to the peristaltic pump.

13. Turn the peristaltic pump on to maintain a flow rate of ~0.25 mL/min. Continue to fill the microcentrifuge tube from Step 3 until it has reached a volume of 0.4-0.5 mL. This will be fraction #1. Continue to collect 4 additional fractions as the extract elutes from the column. Active extract will typically elute in fractions #2 and #3. Avoid prolonged exposure of the labeled extract to light. If possible elute the extract in a darkened cold room while using a flashlight to monitor each fraction. Keep the fractions on ice.

14. Fractions containing the splicing extract should be noticeably yellow in color. Aliquot these fractions in 20-50µL portions, freeze in liquid N₂, and store at -80°C.
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15. Assay each fraction for \textit{in vitro} splicing activity and compare with an unlabeled control extract. Confirm labeling of the SNAP protein by SDS-PAGE of each fraction followed by fluorescence imaging of the unstained gel (\textbf{Figure 2.6}).
Figure 2.5 (preceding page) SEC apparatus used to remove excess bG dye from SNAP-labeled WCE. (A) During equilibration and elution, the column is connected to a peristaltic pump and fractions collected into 1.5 mL microfuge tubes. (B) During WCE loading, a syringe is used to apply pressure to the column to increase the flow rate. Photography credit Robin Davies.

Figure 2.6 Results from SNAP labeling of the spliceosomal Prp16 protein in yeast WCE with bG-DY549. (A) Representative SDS-PAGE gel showing SNAP labeling of Prp16 in WCE and visualized by in-gel fluorescence. Lane 1, protein ladder; lane 2, unlabeled wild-type yeast extract; lanes 3 and 4, fractions obtained by SEC after labeling the Prp16-SNAP extract. (B) The same gel as in A after Comassie blue staining.
2.3.3 Flow chamber cleaning and assembly

1. Place 1-5 large glass coverslips (24 x 60mm) into a clean slide mailer and place 1-5 small glass coverslips (25 x 25 mm) into a second mailer. If slides appear dusty, they can be rinsed with MilliQ H₂O using a squirt bottle beforehand. Due to the likelihood of breaking or dropping a slide during the following steps, it is best to clean more slides at this stage than are needed for subsequent experiments.

2. Fill each slide mailer with a 0.2% v/v Micro-90 solution in MilliQ H₂O. Close the mailer and secure the lid with Parafilm. Float in a sonic water bath for 60 min.

3. Remove the slide mailers from the sonic water bath and pour out the Micro-90 solution. Fill the mailer several times with MilliQ H₂O and pour out the water. Fill the mailer with 100% ethanol, close the lid, and secure with Parafilm. Float in a sonic water bath for 60 min.

4. Remove the mailers from the sonic water bath and pour out ethanol. Fill the mailer several times with MilliQ H₂O and pour out the water. Fill the mailer with 100 mM KOH in MilliQ H₂O, close the lid, and secure with Parafilm. Float in a sonic water bath for 30 min.
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5. Remove the mailers from the sonic water bath and pour out the KOH solution. Fill the mailer several times with MilliQ H₂O and pour out the water. Fill the mailer with MilliQ H₂O, close the lid, and secure with Parafilm. Float in a sonic water bath for 60 min. At the end of sonication, leave the slides submerged in MilliQ H₂O. It is best to proceed directly to derivatization once the slides have been cleaned.

6. Pour out the MilliQ H₂O from the mailer. While wearing clean gloves, carefully remove a slide from the mailer with forceps. Grasp the slide securely by the edges while avoiding contact with the center of the slide and dry with a stream of high-purity N₂ from a “filter gun” attached to a gas cylinder. Once the slide is completely dry, transfer it to a new, dry slide mailer. Repeat for each large slide and smaller coverslip. Up to 5 dried slides can be derivatized in each mailer, but the slides and coverslips should be kept in separate containers.

7. Mix 300 µL of Vectabond with 30 mL of acetone in a plastic 50 mL conical vial. Add the solution to the slide mailer containing the larger slides. Incubate for 5 min.

8. After 5 min, pour the acetone solution back into the 50 mL conical vial and immediately fill the mailer containing the larger slides with fresh MilliQ H₂O. Empty the mailer and fill again with
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MilliQ H₂O. Repeat the water rinse two more times. Leave the mailer empty after the final rinse.

9. Repeat steps 7 and 8 with the mailer containing the smaller coverslips. The same acetone/Vectabond solution can be reused for the coverslips.

10. While wearing clean gloves, carefully remove a slide from the mailer with forceps and dry with a N₂ stream as in Step 6. Once the slide is completely dry, place on a slide holder. We often use either two razor blades or a metal slide holder to secure the slide in an empty pipette tip box while avoiding contact with either the top or bottom of the slide. (Figure 2.7A, B) The slide and/or holder should be gently taped to the empty pipette tip insert rack to prevent movement during subsequent steps or during transportation. The slide can be secured to the holder with tape, a small amount of vacuum grease, or with clear nail polish. To prevent the PEGylated slides from drying out, a small piece of sponge or paper towel soaked in MilliQ H₂O can be placed in the bottom of the pipette tip box to create a humid environment.

11. Using a syringe filled with vacuum grease and fitted with a 200µL plastic pipette tip, draw five grease lanes horizontally across the slide to divide the slide into four chambers (Figure 2.2) Schemes for splinted ligation to prepare fluorescent, biotinylated
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pre-mRNAs. The bold P and OH indicate the 5' phosphate 3' hydroxyl groups of the donor biotin handle and acceptor pre-mRNA, respectively. Sequences shown are for the RP51A pre-mRNA substrate. The biotin handle contains 2' O-methyl residues (lowercase m), a fluorophore (star) attached to a 5-aminoallyl-uridine (5-N-U), and a 3' biotin (Bio). The DNA splints are continuous, but here represented as two distinct portions separated by lines. (A) Splint and junction design for ligation with RNA ligase 2 (RNL2), a double stranded RNA ligase. (B) Splicing and junction design for ligation with RNA ligase 1 (RNL1), a single stranded RNA ligase. Figure adapted from reference (Stark et al., 2006))

12. While wearing clean gloves, carefully remove a coverslip from the mailer with forceps and dry with a N2 stream as in Step 6. Once the coverslip is completely dry, carefully position it above the center of the slide and grease lanes made in steps 10 and 11. Gently push the coverslip into the grease to secure it to the slide and to seal each chamber. (Figure 2.7B)

13. Prepare a fresh solution (10 mL) of 100 mM sodium bicarbonate in MilliQ H2O. Filter the solution through a 0.2 µm syringe filter.
14. Briefly centrifuge a 1 mg aliquot of biotin-PEG to spin any solids down to the bottom of the tube. Dissolve the biotin-PEG in 400 µL of the sodium bicarbonate solution.

15. Briefly centrifuge a 40 mg aliquot of PEG to spin any solids down to the bottom of the tube. Dissolve the PEG in 160 µL of the biotin-PEG solution. Briefly (2s) place the solution in a sonic water bath to help the PEG dissolve. Vortex the solution and then briefly centrifuge. Mix the resulting PEG solution thoroughly by aspirating up and down several times with a pipette.

16. Add the PEG to the side of each chamber. It should enter the chamber by capillary action. Avoid getting any of the PEG solution outside of the chamber. One 40 mg aliquot of PEG is typically enough to derivatize two slides each containing four 20 µL chambers.

17. Incubate the slide at room temperature for at least 3 hours and up to 16 hours.

18. After the room temperature incubation, the slide can be stored at 4°C for up to one week.

2.3.4 pre-mRNA Immobilization and Addition of WCE to the Flow Cell

1. If the derivatized slide has been kept at 4°C, bring the slide to room temperature and allow to equilibrate for ~30-60 min.
Warming the slide to room temperature greatly facilitates removal of the viscous PEG solution in each lane as well as limits the risk of condensation appearing on the slide and interfering with imaging.

2. Prepare 5-10 mL of 2x Wash Buffer (200 mM KPi pH 7.3, 6% PEG 8000, 2 mM DTT) and 2.5 mL of a 2x Splicing Assay Buffer (200 mM KPi pH 7.3, 6% PEG 8000, 2 mM DTT, 10 mM PCA, 5 mM MgCl₂, 2 mM Trolox). It may be necessary to briefly immerse the 2x Splicing Assay Buffer in a sonic water bath in order to fully dissolve the trolox. Alternatively, trolox may be added from a 200 mM stock solution in DMSO or methanol. These stock solutions should be stored at -80°C in single-use aliquots. Filter the 2x Wash and Splicing Assay Buffers through 0.2 µm syringe filters.

3. Prepare a stockpile of “filter paper triangles”. Cut each filter paper into 6-8 triangles, producing pizza slice shaped pieces. Fold each triangle two times by first bringing the pointy end (the bottom of the pizza slice) up to the top and then folding in half again along an axis perpendicular to the first fold. We often prepare many dozens of these folded triangles at once and store them in empty pipette tip boxes.

4. Prepare 2 mL of 1x Wash Buffer using the 2x stock and RNase free MilliQ H₂O. Flush each channel on the slide three times with 100 µL of the 1x Wash Buffer. The washing can be carried out on
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the lab bench using a pipette on one end of the channel and using a filter paper triangle on the other end to draw the liquid through the channel. (Figure 2.7C) The PEG solution is quite viscous and will move slowly through the channel until it has been cleared.

5. The next steps can either be done on the laboratory bench, or if permitted by the microscope configuration, on the microscope stage itself. If work is being done on the microscope, mount the slide to the stage and position the objective appropriately. Extreme care should be taken not to allow liquids to come into contact with the objective or other sensitive components.
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A

B

C
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Figure 2.7 (preceding page) Construction of flow chambers for CoSMoS experiments.  (A) A glass slide is placed onto a slide holder (or a pair of razor blades) and held in place in a pipette tip box with lab tape.  A syringe is then used to draw five thin lines of vacuum grease horizontally across the slide to create four chambers.  (B) A clean coverslip is gently pressed on top of the lanes.  The chamber is completed with the addition of a PEG:PEG-biotin mixture.  The completed slide is also shown schematically in Figure 2.1A.  (C) To wash or add sample to a flow chamber, a pipette is used to dispense fluid to one side of the chamber while the liquid is drawn through to the other side with a small piece of folded filter paper.  Photography credit Robin Davies.

6. Immediately prior to each experiment, streptavidin is added to a slide channel.  For optimal binding of the RNA added subsequently, we recommend adding streptavidin to only one channel at a time just before conducting an experiment in that channel.  Using low adhesion pipette tips and tubes, prepare 50 µL of a 0.2 mg/mL streptavidin solution in 1x Wash Buffer.  Add the entire streptavidin solution to a channel on the slide and draw the solution through using a filter paper triangle.  Let the channel incubate with streptavidin for 2 min and then flush with 200 µL of 1x Wash Buffer.  Proceed immediately to Step 6.

7. Working with low adhesion pipette tips and tubes, prepare a 1 nM stock of the biotinylated, fluorescent pre-mRNA in 1x Wash Buffer that also includes 40 U of RNasin and 0.1 mg/mL nuclease-free BSA.  Store the RNA stock solution on ice.  From this stock solution, prepare 50 µL of a 200 pM RNA stock in 1x Wash Buffer.
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8. Introduce the entire 200 pM RNA stock solution into the slide channel. If this is being done while the slide is mounted on the microscope, the accumulation of the RNA on the slide surface can be monitored in real-time. It is imperative that the microscopic field of view does not become too saturated with RNA molecules and individual molecules are easily resolved and separated from one another. Typically an appropriate surface density is reached in 2-3 min, although this can vary. Stop the RNA accumulation on the surface by flushing the channel with 100 µL of 1x Wash Buffer.

9. Prepare 100 µL of a 1x Splicing Assay Buffer solution by combining the 2x Buffer with ATP (if needed), 0.1 U of PCD, and RNase Free Water. Flush the slide chamber with this solution.

10. At this stage, the slide is ready for WCE to be added. We typically position the slide appropriately for the experiment and optimize laser powers and TIR at this time. Once the microscope has been appropriately configured for the experiment, proceed to step 11.

11. Prepare 100 µL of a splicing assay mixture by combining the 2x Buffer with 2mM ATP (if needed), 35-40µL of the labeled WCE, 0.1 U of PCD, and RNase Free Water. Introduce the assay mixture to the slide channel and begin image acquisition. With practice, this can be done by hand with a deadtime of ~30s. For studying
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events that may occur within that deadtime, a syringe and modified flowcell can be used to pull samples into the slide channel during data acquisition (Crawford et al., 2013; Friedman et al., 2006).

12. We typically acquire data for 30-90 min at intervals of 2-10s between frames (1s/fr). The use of timelapse recording is beneficial for reducing photobleaching of the fluorophores during long experiments and to prevent laser-induced accumulation of fluorescent molecules on the slide surface.

2.4 Notes

1. For efficient ligation, the transcripts should possess homogenous 3' ends and +1 (or greater) non-templated addition productions should be avoided. This can easily be accomplished by using DNA primers containing 2'-methoxy substituents during preparation of the transcription template by PCR (Kao et al., 1999). Alternatively, homogenous ends can be generated by targeted RNaseH cleavage after transcription (Stone et al., 2007).

2. We typically prepare yeast WCE using the method of Ansari and Schwer (Ansari and Schwer, 1995) with the exception of using a ball mill (Retsch) to lyse the yeast cells. The yeast WCE can be aliquoted (1.2 mL) and frozen at -80°C immediately after high-speed centrifugation at 37,000 rpm and before dialysis with no
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effect on splicing activity. For single molecule assays, it is extremely beneficial if the splicing activity of the WCE is as high as possible with at least 20% of the pre-mRNA being converted to mRNA in 30-45 min at room temperature for yeast WCE.

3. SNAP tag substrates can be resuspended in DMSO, aliquoted, and stored at -20°C. These aliquots retain labeling activity for many months. It is critical that the concentration of the BG substrate be quantified accurately by UV-Visible spectroscopy so that neither too much nor too little is added to the yeast WCE during labeling.

4. Great care must be taken in choosing the appropriate combination of oxygen scavengers, reducing agents, and triplet quenchers for the single molecule experiment. These components should be tested for possible inhibition or interactions with the biomolecules under study including detrimental RNase or DNase activity. Additionally, some oxygen scavenging systems may influence the pH of poorly buffered assay mixtures and this should be studied prior to setting up the single molecule assay.

5. To maintain surface attachment chemistry, both the Vectabond and PEG solutions should be carefully aliquoted. Vectabond can be stored in 300 µL aliquots in 1.5 mL microfuge tubes that have been backfilled with dry nitrogen or argon. We store the Vectabond at room temperature and protected from light. Aliquots retain
activity for several weeks; however, we only aliquot one stock solution of Vectabond at a time. It is critically important that PEG and biotin-PEG aliquots be made when the bottles are first opened. We typically allow the stock bottles to come to room temperature (to avoid condensation) and make ~40 mg aliquots of PEG and ~1 mg aliquots of biotin-PEG in separate 0.5 mL microfuge tubes. We backfill each tube with dry nitrogen and store at -20°C in a container with a tight fitting lid and with desiccant. These aliquots will retain reactivity for many months.
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3.1 Introduction

Pre-mRNA splicing is an essential process in eukaryotic gene expression. This process is catalyzed by the spliceosome, a large and highly dynamic macromolecular machine composed of multiple RNAs and proteins. The spliceosome assembles stepwise on the pre-mRNA substrate as a series of complexes defined by their snRNP composition: A, B, C, P, ILS.

Spliceosome assembly and disassembly require ATP hydrolysis and are modulated by trans-acting factors. These splicing trans-factors were first identified in a yeast genetic screen for splicing deficiencies (Brawerman et al., 1972). Nearly all transitions and steps in splicing are governed by DEAH-box proteins, a key subset of the SF2 RNA-dependent ATPases. These proteins include Prp2, Prp16, Prp22, and Prp43 and are highly conserved and act at sequential steps in the splicing process. These DEAH-box proteins are thought to use their ATPase/helicase activity to remodel the spliceosome at distinct stages by disrupting RNA-RNA or RNA-protein interactions.

One of the most studied DEAH-box family members is Prp22, which acts during the second step and promotes release of the spliced mRNA from the spliceosome. Its function during the second step is ATP-independent and requires the ordered binding of the proteins Slu7 and Prp18 to the spliceosome (James et al., 2002). Based on data from crosslinking and RNase H protection assays, Beate Schwer has proposed a model in which Prp22p facilitates mRNA
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release by binding downstream of the 3’-exon after exon ligation (Schwer, 2008). Prp22p then presumably uses its ATPase activity to disrupt RNA-RNA and RNA-protein interactions between the mRNA and U5 snRNP components (U5 snRNA and Prp8), although how it does this and what exact interactions it disrupts are not clear.

There remain several open questions concerning these DEAH-box proteins and their roles as in splicing and proofreading. Previous in vitro depletion and reconstitution assays have shown at what major transition steps they are required. Yet little is known about when and where they are binding with the spliceosome. The kinetic details of the transient interactions of the DEAH-box proteins with the spliceosome have not been observed, let alone analyzed. In particular, it is not known how their remodeling actions are timed or specified to their associated splicing events. It is also not clear what are their actual RNA landing sites and what are the substrates for their remodeling activity. Finally, the nature of the discard step for suboptimal substrates has not been fully addressed. Discard could entail repeated cycles of rejection and testing of substrates, or involve a spliceosome disassembly pathway in coordination with Prp43 (Mayas et al., 2010).

The Moore laboratory has recently developed CoSMoS for monitoring thecomings and goings of individual spliceosomal subcomplexes on surfaced tethered pre-mRNA molecules in crude cell lysates. Previous analyses have revealed that every major step in spliceosome assembly is reversible and that
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catalytically active spliceosomes can be assembled by multiple pathways (Hoskins et al., 2011; Shcherbakova et al., 2013). Here I describe the application of CoSMoS to follow the dynamics of Prp22 association with spliceosomes formed on pre-mRNA molecules capable of completing both steps of splicing. The experiments detailed in this chapter demonstrate my ability to watch Prp22 interact with spliceosomes during splicing. Analysis of these experiments revealed an apparent binding stoichiometry of 1 Prp22 protein per spliceosome, and the binding order of Prp22p relative to the NTC (activation) and intron release (splicing).

3.2 Results

3.2.1 Preparation of fluorescently labeled Prp22 for CoSMoS experiments

To initiate our Prp22 studies, I used homologous recombination to create a haploid yeast strain (Jones, 1977) in which all Prp22 molecules carried a fSNAP tag on their C-termini via a (GSG)$_2$ linker (Sun et al., 2011) (Figure 3.1A). This tag was introduced into both a strain containing no other tags and a strain carrying C-terminal ecDHFR tags tethered to the Cef1 and Ntc90 components of the NTC complex (Hoskins et al., 2011). Consistent with previous studies showing that GFP and TAP tags are allowable on Prp22 (Ghaemmaghami et al., 2003; Huh et al., 2003), our tagged strains grew with the same rates as to their respective parental strains (doubling time ~1.4 hr). However, whole cell extracts from the Prp22-fSNAP strains exhibited a defect in step two splicing kinetics in
ensemble splicing reactions relative to the respective parental strains (Figure 3.1B). This defect was characterized by an excess in lariat intermediate and up to ~2 fold less splicing activity compared to the parental strain. This second step defect was also observed by an mRNA displacement assay (Schwer and Gross, 1998) (Figure 3.1C) wherein the lariat intermediate was observed in both the heavy (cosedimented with the spliceosome) and light fractions (released from the spliceosome) from the glycerol gradient ultracentrifugation.

While this second step kinetic defect is significant, the overall splicing activity was within the range observed for strains used in previous CoSMoS splicing experiments (Anderson and Hoskins, 2014; Hoskins et al., 2011). Therefore it didn’t preclude using Pr22-fSNAP for preliminary CoSMoS experiments in that the yeast strain grows without issue and still retains an ability, albeit lessened, to splice pre-mRNAs and displace spliced mRNA from the spliceosome in vitro. Indeed, using CoSMoS it may be possible to identify and distinguish the on and off splicing pathways associated with this strain. Finally, splicing activity as determined by an in vitro splicing assay is highly dependent upon the concentration of the whole cell extract being used. Recent splicing assays using labeled Prp22-fSNAP extracts that are up to 4-fold more concentrated than extracts typically used for CoSMoS experiments (~80 vs 20 mg/mL protein concentration by Bradford assay) show qualitatively a much higher activity (25-40% splicing vs 15%) and faster splicing kinetics (Figure 3.1D).
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To generate labeled extracts for single molecule studies, I used the Snap-tag dye substrates benzyl-guanosine-DY549 (BG-DY549; green) or BG-CF633 (red) to label Prp22-fSNAP. Because *S. cerevisae* splicing extracts presumably lose activity over time, I had to determine optimal labeling conditions that had enough dye to achieve near quantitative labeling within 30 minutes at 25°C, but not so much that I could not remove it later. A titration revealed that 1.2uM or 750 nM BG-DY549 or BG-CF633 was sufficient to saturate labeling of the regular or fast versions of Snap-tag within 15 min (**Figure 3.1E**). As detailed in section 2.3, this dye concentration was readily reduced to that usable for CoSMoS experiments (i.e., <50 nM) using size exclusion chromatography.

**Figure 3.1 (below):** Preparation of labeled whole cell yeast extracts to be used in CoSMoS splicing experiments.  
A  Scheme detailing preparation of labeled yeast extracts starting with protein engineering in yeast to lysis and labeling of the strain. The size exclusion column is critical for removing the excess dye from the labeling reaction.  
B  *In vitro* splicing assay comparing activity of a wild-type to a Prp22-Snap extracts; the Prp22 strain splices but less so.  
C  Assay for mRNA release from the spliceosome using glycerol gradient ultracentrifugation.  
D  In vitro splicing assay of concentrated Prp22-fSnap extracts (~60-80 mg/mL).  
E  SDS-PAGE of a Prp22-fSnap labeling titration to determine the minimal amount of Snap-tag dye to be used for saturated labeling in 15 min.
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A

<table>
<thead>
<tr>
<th>prp22</th>
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genticly encode orthogonal tags

Haploid Yeast Strains

lyse yeast cells

Whole Cell Extract

1. label Snap-tag
2. size exclusion

CoSMoS Splicing Experiment

1. label DHFR
2. add to splicing assay
3. add to slide-attached fluorescent RNA

B

In Vitro Splicing Assay of Wild Type and Prp22-Snap Yeast Extracts

C

Assaying Prp22 Functionality: miRNA Release from the Spliceosome

D

In Vitro Splicing Assay of High Concentration Prp22-Snap Whole Cell Extracts

E

Normalized Splice Trace
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3.2.2 Prp22 binding dynamics and stoichiometry

Ensemble studies have shown that Prp22 only stably associates with late stage spliceosomes. To observe both Prp22p binding dynamics and stoichiometry, I ran a 3-color experiment in which blue-labeled RNA locations were monitored for colocalization of green and red Prp22 events (Figure 3.2A). For these experiments I used an extract wherein individual Prp22 molecules carried either a green or a red dye. To account for non-specific binding a dark control was performed. The dark control is from the same experiment and consists of using a similar number of locations that do not have blue RNA spots (Friedman and Gelles, 2015). Both pre-mRNA and Prp22 spots were detected using a spot fitting algorithm that finds and centers spots within an image after background reduction (Blair and Dufresne; Crocker and Grier, 1996). A colocalized event was defined as a green or red spot of a specified brightness landing within 1.5 pixels of the center of the pre-mRNA location. Fluorescent traces were constructed by mapping and drift-correcting pre-mRNA locations from the blue field to the green and red Prp22 fields and integrating over the course of the experiment (up to ~60 min). Note, due to poor low signal/noise with this particular experiment, both integrated intensities and spot detection were performed with averaging every 5 frames. As a built-in control, ‘dark’ locations devoid of a pre-mRNA spot were also integrated and spots detected for Prp22 signal. Example traces for different pre-mRNA locations are shown in Figure 3.2B.
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To facilitate visual analysis of these data, I prepared rastergrams. Rastergrams are a visual tool in which individual traces are reduced to digital signals represented as colored bars. Each row represents a different RNA location within the experiment and are stacked onto one another. Rastergrams as a visual tool were originally conceived by Phil Zamore and have been used to visualize CoSMoS experiments of spliceosome assembly and RNAi (Salomon et al., 2015; Shcherbakova et al., 2013) (Figure 3.2C).

The rastergrams of my 3-color Prp22 experiment show the dynamics of Prp22 landings, with multiple comings and goings, consisting of both long and short events (Figure 3.2D). The white space of the rastergrams show the absence of detected Prp22 binding activity, with a majority of pre-mRNA locations not having any activity at all. Note this is typical of CoSMoS splicing experiments and a feature of the low activity of in vitro splicing assays in general.

If I compare RNA locations to dark locations (Figure 3.2E), there are about twice the number of total events at RNA locations (253 vs 101 events >5 sec). Thus, like the snRNPs, Prp22 association appears to be specific to pre-mRNA locations and its association is dynamic with multiple comings and goings.

**Figure 3.2 (below):** Only 1 Prp22 protein binds to the spliceosome at a time. A Scheme for using CoSMoS to determine Prp22 binding stoichiometry; Prp22 has been separately labeled with red and green dyes. B Example 2-color traces showing distinct Prp22 proteins bind close in but not at the same time. C A trace can be flattened into a 2D digital image and constructed into a rastergram; only the binding events are used and depicted with their respective colors. D-ERastergrams for the RNA and Dark control experiments; red or green bars show single Prp22 binding events, blue bars show overlaps, white area is absence of detected events.
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Previous analysis of snRNP and NTC binding revealed no simultaneous binding events for any single species (i.e., subunit stoichiometries = 1;) (Hoskins et al., 2011). This is also most likely true for Prp22. By encoding different species with different colors, rastergrams allow one to easily visualize the kinetics of appearance and disappearance of multiple colored species on many different RNAs at once (Figure 3.2D). Although I cannot be certain that every Prp22 molecule carried a dye in that experiment, I only saw 7 instances of a brief overlap (total dwell time ~ 18 min) out of 253 total green and red Prp22 events (total dwell time ~ 295 min). For the dark control data set, there only 5 brief overlaps (total dwell time ~4 min) out of 101 total green and red Prp22 events (total dwell time ~ 75 min). Note that the frequency of simultaneous green and red Prp22 binding is about the same for the RNA and dark control data sets (~0.05%). Given this I conclude the Prp22 overlaps present in the RNA experiment can be ascribed to random background events. Interestingly, while not overlapping, the red and green Prp22 events are often within <10 sec, indicating distinct Prp22 molecules binding within short order of one another. Thus my data are consistent with Prp22 acting as a monomer, for which there is only a single binding site on the spliceosome.

3.2.3 Prp22 relative to spliceosome activation

Previous ensemble studies have indicated that Prp22 stably associates with post-activation spliceosomes (i.e., C-complex) (Fabrizio et al., 2009; Lardelli et al., 2010). But exactly when Prp22 first associates is not known. All of these
complexes contain the NTC, the acquisition of which is the last major step in spliceosome assembly. Along with displacement of the U1 and U4 snRNPs and Brr2-mediated annealing of U2 and U6 snRNAs, stable NTC association is a hallmark with activating the spliceosome for first step chemistry. While previously little was known about the NTC structure and where it resides within the spliceosome, there have been some interactions (both proteomic and genetic) between the NTC and Prp22. Quite recently a high resolution cryo-EM structure of the *S. pombe* post-activation spliceosome (most likely a mixture of C, P and ILS-complexes) has been reported which includes a NTC structure (Yan et al., 2015). The NTC structure occupies a central space above the U5 snRNP and catalytic center of the spliceosome, providing a massive extended framework of ~ 200 Å² and 170Å thick. These structural features suggest perhaps NTC acts as a recruiting platform for Prp22 and other splicing factors.

To determine when Prp22 binds relative to the NTC, I performed 3-color CoSMoS experiments with blue-labeled wild type RP51A pre-mRNAs, green-labeled Prp22 and red-labeled NTC (*Figure 3.3A*) in the presence or absence of ATP. Extracts depleted of ATP serve as a negative control for splicing and spliceosome activation which require the addition of ATP (Liao et al., 1992; Tatei et al., 1989). After recording the RNA locations, the Prp22 and NTC labeled WCE was added to the slide chamber and Prp22 and NTC events were recorded simultaneously and continuously at a frame rate of 1 frame/sec. To visually analyze these experiments I prepared rastergrams as shown in *Figure 3.3B*. 
The rastergrams for 240 RNA locations, 240 dark locations and 287 No ATP locations are shown in Figures 3.3C-E. NTC signals lasting more than 5 secs/frames were detected at 86 of the RNA+ATP locations (36%), but only 20 of the dark locations (8%) and 20 of the RNA No ATP locations (7%). Similarly, Prp22 signals lasting more than 5 secs/frames were detected at 78 of the RNA locations (32%), but only 19 of the dark locations (8%) and 60 of the RNA No ATP locations (20%). Finally, 56 RNA locations (23%) exhibited simultaneous occupancy of both NTC and Prp22, compared to only 3 dark locations (1%) and 4 RNA No ATP locations (1%). The relatively high number of Prp22 events in the No ATP control experiment is surprising. However, these events appear to be short and could represent a high background from that experiment. This illustrates the background and potential variability associated with CoSMoS experiments. Nonetheless, in general both NTC and Prp22 exhibited many more binding events at RNA locations in an ATP dependent manner, with a majority of RNAs that bound NTC also binding Prp22.

Figure 3.3 (below) Prp22 dynamic binding to the spliceosome is RNA and ATP dependent. A 3-color CoSMoS experiment for watching Prp22 (green) and NTC (red) land at pre-mRNA locations (blue). B Example raw intensity traces of Prp22 and NTC binding to a pre-mRNA. The gallery of spots (above) shows the appearance of Prp22 spots (top row) and later simultaneous disappearance with NTC spots (bottom row). The middle plot shows the same traces but using the spot brightness information from the automated spot picker (Blair and Dufresne). The dashed line shows if the binding event was colocalized to the RNA location, which is where the colored bars of a rastergram depict. C-E Unsorted rastergrams of the RNA, Dark control and No ATP control Prp22/NTC experiments. NTC, Prp22 and both binding are depicted by red, green and blue bars, respectively.
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While the 3-color rastergrams are informative in that they show the entirety of a CoSMoS experiment, it is difficult to directly compare the relative timing of the different events. Andrew Franck has made improvements to the rastergrams by adding the abilities to remove empty RNA locations devoid of events, sort and align the 3-color rastergrams. Recently I added a so-called wiggle plot option, which displays the total number of events over time.

To examine the time of appearance for Prp22 versus NTC, I aligned and sorted the rastergrams by duration of the first Prp22 event. This rastergram still conveys the whole experiment, but more readily shows the amount of binding activity. For the RP51A +ATP experiment the rastergram shows only about a third of the RNA locations have Prp22 and NTC activity (Figure 3.4A). This is greater than the apparent activity of the dark control data, which is <10% (Figure 3.4B). Interestingly, the No ATP rastergram again shows little NTC activity, but about 20% RNAs have green Prp22 events (Figure 3.4C). However, examination of the corresponding wiggle plot shows the majority of these green Prp22 events are relatively short lived (<2 min) compared to the +ATP experiment (<10 min). It appears the No ATP experiment had a significant green Prp22 background, however, this was not correlated with NTC activity (i.e., only 4 RNAs with Prp22/NTC overlaps).

Figures 3.4D-F show the same rastergrams as before but with empty RNA locations removed. From these rastergrams I made the following observations:

1. For the RP51A +ATP experiment, of the 78 RNAs that got a Prp22 signal, 61
(78%) also had a NTC signal. However, of the 60 No ATP RNA and 19 dark locations that had a Prp22 signal, only 6 (32%) and 4 (7%) also had a NTC signal. Thus there is a much higher correlation between Prp22 and NTC occupancy on pre-mRNAs than at dark or No ATP RNA locations. (2) Of the 61 +ATP RNA molecules having both Prp22 and NTC signals, 56 exhibited one or more periods of simultaneous occupancy. (3) For these cases where there was an overlap in NTC and Prp22 binding, there was a strong tendency of NTC to appear first, followed by a mean delay time of <5 minutes until Prp22 appears (estimated from the wiggle plot, Figure 3.4D). Thus my data strongly suggest that stable association of Prp22 with spliceosomes requires prior arrival of the NTC.

Figure 3.4 (below) NTC binding appearance tends to precede Prp22 appearance. A Rastergram and wiggle plots aligned by the first Prp22 event and sorted by its duration. The colored bars are as before (red=NTC, green=Prp22, blue=both), grey space indicates no data areas. From this rastergram can estimate the amount of Prp22 activity. The wiggle plot is simply a sum of the Prp22 and NTC events plotted over the experiment time. B-C Rastergrams sorted and aligned as in A, but for the Dark locations and No ATP control experiments. D-F Same rastergrams as in A-C but with the ‘empty’ RNAs devoid of Prp22 events removed. (The rastergram code was developed and kindly shared by Andrew Franck. Idea of using wiggle plots to sum up the rastergrams is from Danny Crawford.)
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I also ordered the rastergrams by the time of the last Prp22 event (Figures 3.5A-F). For the +ATP RNA rastergram, this shows some NTC events extend past the last Prp22 event but also some NTC events disappear simultaneously with those of Prp22 (Figure 3.5D). Analysis of the underlying data revealed that of the 56 RNAs with Prp22 and NTC overlaps, the Prp22 signal disappears either prior to (33 times, 59%) or coincident with (22 times, 39%), but only rarely (1 time, 2%) after NTC disappearance. I also observed half of the 63 overlaps consisted of 1 Prp22 event per NTC event (49%). The remaining half consisted of instances of 2 (22%), 3 (11%), 4 (10%) and more than 5 (9%) Prp22 events overlapping with 1 NTC event. These data suggest that activated spliceosomes can bind and release Prp22 multiple times prior to NTC departure. Once NTC disappeared, the Prp22 signal rarely reappeared (3 times) unless the NTC also reappeared (8 times). Finally, of the 22 times the Prp22 signal disappeared simultaneously with that of the NTC, the majority (17) did not have any subsequent binding events of either Prp22 or the NTC.

Figure 3.5 (below) Ordering of disappearance of Prp22 and NTC events. A-C Rastergrams and wiggle plots for the RNA, Dark locations, and No ATP control experiments were aligned by the last Prp22 event and sorted by its duration. The colored bars are as before (red=NTC, green=Prp22, blue=both), grey space indicates no data areas. D-F Same rastergrams as in A-C but with the ‘empty’ RNAs devoid of Prp22 events removed.
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While the visual patterns of Prp22p and NTC events by rastergrams are striking, to gain further insight into the ordering kinetics, I quantified the difference in appearance and disappearance times for Prp22p and NTC (tPrp22-tNTC). I restricted this analysis to overlapping events of Prp22 and NTC (i.e., simultaneous binding of the pre-mRNA by Prp22 and NTC) and calculated the difference in start and end times for such events (Figure 3.6A). As shown in a histogram of the differences in appearance times (Figure 3.6B), the majority were positive (90%). This suggests a binding order of NTC first, followed by Prp22, confirming the visual trend observed in the rastergrams. Note that the delay in appearance of Prp22 after NTC binding was ~2 min, which is consistent with previous CoSMoS-FRET study of pre-mRNA splicing dynamics (Crawford et al., 2013). An ordering of the NTC followed by Prp22 does not disprove the NTC acting as a recruiting element for Prp22 per se and suggests Prp22 interacts with spliceosomes after activation (Fabrizio et al., 2009). Given the lack of simultaneous appearance (tPrp22-tNTC = 0, 4%), a pre-formed Prp22-NTC complex binding to the pre-mRNA is unlikely.

I also examined the order of event disappearance for Prp22 and NTC overlaps and found most of the differences were negative (62%) (Figure 3.6C,D). This would seemingly suggest Prp22 tends to dissociate prior to the NTC. Yet a significant fraction (27%) of the Prp22-NTC overlaps resulted in a simultaneous disappearance of both Prp22 and NTC signals (tPrp22-tNTC = 0).
This would be representative of the pre-mRNAs that bound both Prp22 and NTC which later dissociated at the same time.

A potential issue with this overlap difference analysis is if one of the two events tends to have longer dwell times, then by chance alone I would expect the longer event to appear first. In the case of these experiments, NTC tends to have longer dwell times than Prp22 (mean dwell times of ~3 vs 1 min respectively), so the null hypothesis is the ordering of NTC before Prp22 could be due to chance. To test this I scrambled the experiment such that the Prp22 and NTC events were drawn from different RNA locations. Overlaps from this scrambled data set were detected and quantified as before. This scrambling procedure was repeated until the scrambled set had a similar or greater number of overlaps (n~100) as the original experiment. As expected, the overlap analysis for the scrambled control shows NTC tends to precede Prp22, but not to the same extent as the experimental data (Figure 3.6B, red histogram). Note the scrambling control has a larger number of negative arrival events (36%) versus the experiment (6%). I conclude that Prp22 tends to arrive after NTC and does not appear as a pre-formed complex (i.e., simultaneous appearance).

The simultaneous disappearance I observed for Prp22 and NTC is more striking in light of the scrambling control. Here the scrambled data set shows 3% simultaneous events versus the 27% for the experiment (Figure 3.6D, red histogram). Note that a disappearance in fluorescent signal can be from a dissociation or photobleaching event. I did not perform a photobleaching control.
and therefore cannot estimate the contribution of photobleaching to signal
disappearance. However, the acquisition conditions of this experiment are
similar to previous studies with known minimal photobleaching (Hoskins et al.,
2011; Shcherbakova et al., 2013). Furthermore, the simultaneous
photobleaching of 3 unique fluorophores on 2 different proteins (1 on Prp22 and
2 on the NTC) is extremely unlikely and can effectively be ruled out. Altogether,
this strengthens my observation that a subset of pre-mRNAs that have Prp22
and NTC bound at the same time show simultaneous departure of Prp22 and
NTC. It is tantalizing to speculate this observation could be ascribed to either
splicing (i.e., mRNA release from spliceosome) or proofreading (dissociation of
the spliceosome after unsuccessful splicing).

Figure 3.6 (below) Determining the difference in appearance and
disappearance times for Prp22 and NTC overlapping events. A Detailed trace of
overlapped Prp22 and NTC binding at the same RNA location. The difference in
appearance times is marked with the solid and dashed black lines. In this
example, Prp22 appears well after NTC, so the difference (tPrp22 – tNTC) is
positive. B Histogram of the delays in Prp22 and NTC appearance times. Note
this analysis is restricted to only overlapping Prp22 and NTC events (i.e., Prp22
and NTC bound to the RNA at the same time). And of those overlaps, only the
outer pair of events are used for the calculation (i.e., first and last Prp22 event).
The open red histogram is from a scrambled control of the same data. The
scrambling means different Prp22/NTC overlaps are generated by switching RNA
locations. C Same traces as in A but now showing how difference in Prp22 and
NTC disappearance times are determined. In this example, Prp22 and NTC
disappear simultaneously, so the difference is 0. D Histogram of the difference
in Prp22 and disappearance times. Open red histogram is from scrambled
control.
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A

B

C

D
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For these Prp22-NTC overlap events I also examined the wait and dwell times of Prp22 (Figure 3.7A). The wait time of the first Prp22 event is simply the time after the start of a NTC event before Prp22 first appeared. The first Prp22 event occurs on average ~3 minutes after the start of the NTC event (Figure 3.7B), which is similar to what the rastergrams and overlap analysis show. In the case of multiple Prp22 binding events, the last event mean wait time of 33 seconds is much shorter (Figure 3.7C). The longer initial wait time for Prp22 appearance after NTC binding upstream is consistent with a delay presumably due to upstream spliceosome remodeling and splicing events.

I also examined the mean dwell times for Prp22 events overlapping with NTC. The mean dwell time for all Prp22 events in overlaps of NTC is 74 sec (Figure 3.7D). The dwell times for first or last Prp22 events did not differ with means of 70 and 77 sec, respectively (Figure 3.7E and 3.7F). Without knowledge of the photobleaching rate, it is difficult to ascribe meaning to the similar dwell times. Assuming photobleaching is not a significant factor, the similar dwell times would suggest the off rate of Prp22 is similar regardless of when it binds relative to NTC.

Figure 3.7 (below) Detailed examination of the wait and dwell times of Prp22 binding to the spliceosome. A Scheme showing which Prp22 wait and dwell times were analyzed; these are only from Prp22/NTC overlapping events. B Histogram of wait times for the first Prp22 binding event after NTC binding. C Histogram of the wait times for the last Prp22 binding events; this is only for when there are multiple Prp22 events per 1 NTC event. D-F Histograms of the dwell times of all, first or last Prp22 events, respectively.
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3.2.4 Prp22 and NTC binding relative to intron release

Once exon ligation has occurred, Prp22 uses its ATPase activity to
dissociate the lariat intron-containing spliceosome from the spliced exons.
Immediately prior to this event, Prp22 is bound to both the splicing machinery
(presumably via its N-terminal domain; (Schneider and Schwer, 2001)) and the
exonic region 15-20 nts downstream of the exon-exon junction (Schwer, 2008).
Whether Prp22 first releases the spliceosome, first releases the spliced exons,
releases both stochastically or releases both simultaneously is not known. It is
also unknown how many Prp22 binding and release events are required to bring
about dissolution of the association between the spliceosome and spliced mRNA.
Finally it is not clear what happens in the event of incorrect splicing – does Prp22
also promote release from the spliceosome of intermediates that have not
completed the second step of splicing?

To address these questions, I next performed a three-color experiment
using green-labeled Prp22, red-labeled NTC and RPS30A pre-mRNA containing
7-12 blue (Alexa488) dyes within the intron (Figure 3.8A). Note this RNA was
designed and prepared similarly to that used to good effect by Inna
Shcherbakova (Shcherbakova et al., 2013). The second step defect associated
with tagging Prp22 leads to slower second step kinetics (measured ensemble)
but there is still some splicing activity. At the single molecule level then it should
be possible to observe both on and off pathway trajectories for Prp22, NTC and
the labeled intron (Figure 3.8B).
Figure 3.8 Framing Prp22 binding dynamics to spliceosome assembly and intron release.  

A  Scheme for CoSMoS experiment combining labeled Prp22/NTC extract with a RPS30A pre-mRNA containing a labeled intron.  

B  Scheme showing possible splicing or discard pathways observed from this CoSMoS experiment.  Spliceosome structure image from the recent cyro-EM structure of the *S. pombe* spliceosome (Yan, et al., 2015).
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I first assessed the RPS30A pre-mRNAs for specificity and activity of Prp22 and NTC binding. In this manner, the RPS30A pre-mRNA with a labeled intron reports only its location and not intron release. This is similar to the RP51A pre-mRNA substrate used in the above experiments. Figure 3.9A-D show the unsorted rastergrams and wiggle plots for 600 RNA locations and 600 dark locations, both with and without ATP. In general, the results are similar to those for the RP51A experiments, in that the binding of Prp22 and NTC are dynamic, RNA-specific and ATP-dependent. In roughly comparing the peak heights of the wiggle plots, it appears all of the control experiments have about half the Prp22 and NTC events as the +ATP, +RPS30A pre-mRNA experiment. There does seem to be a nonspecific background for both the green Prp22 and red NTC signals in the control experiments, but seems to be mostly comprised of short-lived events.

Figure 3.9 (below) Binding of Prp22 to RPS30A containing pre-mRNA spliceosomes is RNA specific and ATP dependent. A-D Wiggle plots and unsorted rastergrams for Prp22 and NTC binding to RPS30A RNA and dark locations, both with and without ATP.
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To examine the activity of the labeled RPS30A pre-mRNA, I aligned the rastergram of the RNA locations with ATP by the time to the first NTC event and normalized to the active fraction of RNA (i.e., number of RNAs that bound NTC) (Figure 3.10A). I compared this to the same type of rastergram for the RP51A experiment described above (Figures 3.10B). While both RPS30A and RP51A had about the same active fraction (~0.3), the binding of NTC to RPS30A was faster than RP51A. Note this observation is similar to the previous findings in which RPS30A had faster splicing kinetics than RP51A (Shcherbakova et al., 2013). Further note this comparison between activities and kinetics of RPS30A and RP51A is confounded by the different whole-cell yeast extracts used in the experiments. While the extracts are from the same tagged Prp22/NTC yeast strain, they differ greatly in protein concentration: ~20 vs ~80 mg/mL for the RP51A and RPS30A experiments, respectively. This concentration difference is from an improvement in the extract preparation (i.e., reduction in the resuspension volume of the yeast pellet prior to lysis) and most likely accounts for some of the difference in splicing kinetics between RPS30A and RP51A.

I next examined the binding order of Prp22 and NTC to the RPS30A pre-mRNA. Figure 3.10C shows the rastergram of the RNA locations with ATP sorted and aligned by the first Prp22 event. In general and similar to the trend observed with RP51A, Prp22 tends to appear after NTC, but with less of a delay as seen with RP51A (~1 vs 2-3 min, compare to Figure 3.4A). When sorted and aligned by the last Prp22 event, the rastergram (Figure 3.10D) showed a general
order of disappearance of Prp22 followed by NTC and many simultaneous
disappearance events of Prp22 and NTC, again similar to the results with RP51A
(compare to Figure 3.4D).

Figure 3.10 (below)  Prp22 and NTC exhibit similar binding patterns for RP51A
and RPS30A pre-mRNAs.  A  Wiggle plot and rastergram sorted for time to first
NTC event in the RPS30A experiment.  The rastergram has been normalized to
the number of 'active' RNAs (i.e., RNAs that bound NTC, ~30%) and x-axis
scaled past the actual experiment time to facilitate comparison with the RP51A
rastergram.  B  Wiggle plot and rastergram sorted for time to first NTC event in
the RP51A experiment.  This rastergram was also normalized to the number of
active RNA (~30%).  C-D  Wiggle plots and rastergrams for the RPS30A
experiment sorted and aligned by first and last Prp22 events, respectively.
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In this experiment splicing or more specifically intron release is defined as loss of the fluorescent intron signal. To examine intron loss due to photobleaching a ‘No ATP’ control experiment was performed in which the extract was depleted of ATP as before by adding glucose to the WCE. To account for RNA degradation, RNA spots were quantified at the beginning and end of the experiment in fields that were exposed to the lasers used in the experiment or not. The difference in the fraction remaining RNA spots in the ‘+ATP’ and ‘No ATP’ experiments would be due to splicing and photobleaching. In this case photobleaching is the dominant process for intron signal loss in that there is no difference between the ‘+ATP’ and ‘No ATP’ experiments (Figure 3.11A). Even restricting analysis by selecting for brighter initial RNA spots (i.e., theoretically more fluorophores) did not show a difference between the ‘+ATP’ and ‘No ATP’ experiments. This suggests that the labeled intron does not contain as many fluorophores as determined from UV/Vis quantification of the labeled pre-mRNA.

Despite the apparent failure of the labeled intron to faithfully report on splicing, I examined individual RNAs that did have a Prp22, NTC and intron signal. Note this is an advantage of single molecule experiments: even though the experiment failed at the ensemble level, can still look at individual molecules to provide some insight.) My analysis suggests some of the RPS30A pre-mRNAs with an intron signal still present when Prp22 and NTC colocalize do not splice (i.e., intron spots still remain after simultaneous disappearance of
Prp22/NTC) (Figure 3.11B). This result was counter to the expectation of simultaneous Prp22/NTC disappearance due to successful splicing. Interestingly, after loss of Prp22 and NTC, I do not observe subsequent binding events, possibly suggesting these subset of pre-mRNAs are no longer competent for spliceosome assembly and splicing. In some cases, RNA degradation can be ruled out since the intron signal remained observable at the surface for several minutes after Prp22/NTC disappearance.

In the other case of intron signal loss during a Prp22/NTC overlap (Figure 3.11B, right), since loss of fluorescence is only a single step I cannot distinguish between photobleaching and intron dissociation. Since the time resolution for the intron is poor (acquired 1 sec/frame every 2 min) relative to that of Prp22 and NTC (acquired continuously at 1 sec/frame), I also cannot remark on the exact timing of intron signal loss relative to the simultaneous disappearance of Prp22 and NTC. However, it does hint at what possible splicing would look like under these imaging conditions.

Figure 3.11 (below) Assessing the fate of the RPS30A intron. A Intron spot survival curves for the RPS30A experiments. For each plot, the fraction of remaining intron spots over time is plotted for the +ATP and No ATP experiments. These plots are progressively filtered for increasing initial spot brightness (0 to 5000 pixels). B Scheme for possible discard or splicing events after Prp22 and NTC binding. Below are 2 example traces showing spot brightness over time for the RPS30A labeled intron, Prp22 and NTC. Note in both cases the simultaneous disappearance of Prp22 and NTC.
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3.3 Discussion

The CoSMoS technique has been previously used to study pre-mRNA splicing and spliceosome assembly pathways using a combination of fluorescently labeled pre-mRNAs and proteins in snRNPs and NTC complexes (Crawford et al., 2013; Hoskins et al., 2011; Shcherbakova et al., 2013). This work expands the CoSMoS technique for studying individual proteins involved in splicing while retaining the advantages of using multiple labeled complexes and pre-mRNA. This chapter reports on a series of CoSMoS experiments that observed the dynamics of the DEAH-box protein Prp22 relative to spliceosome assembly and pre-mRNA splicing events. These experiments used 3 colors to directly watch Prp22 interact with spliceosomes during splicing and specifically determine its binding stoichiometry, and binding order relative to the NTC and to intron release from a surface-tethered pre-mRNA. Unexpectedly, my results suggest that our current model of the ordering of spliced exon release and spliceosome disassembly is incomplete. Although additional experiments are required, my data indicate that both Prp22 and the NTC can depart prior to the intron product. I propose alternate disassembly pathways that should be tested in the future.

3.3.1 Preparation of fluorescently labeled Prp22 for CoSMoS experiments

CoSMoS splicing experiments require a combination of fluorescently labeled pre-mRNAs and whole-cell extracts. Fluorescently labeling of specific proteins in WCE is a challenge that was solved by using orthogonal tagging
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methods, specifically Snap-tag and DHFR-tag systems (Calloway et al., 2007; Sun et al., 2011). All of the DEAH-box proteins involved in splicing are essential and therefore a rough but absolute benchmark for tagging is yeast cell viability. Yeast strains wherein the Prp22 gene is replaced with a Prp22-c-terminal Snap-tag fusion are viable and grow at the same rates as wild type. Nonetheless, in vitro assays for splicing activity and mRNA release revealed an ~2-fold kinetic defect in the second step in splicing compared to wild type extract. Yet there both splicing and mRNA release do occur in vitro, and the wild type-like growth rate of the tagged strain indicates that any kinetic defect is not rate limiting for cell growth. It is also possible this defect could be specific to the model RP51A pre-mRNA substrate used in the in vitro splicing assays, but this was not examined with other pre-mRNAs. It is interesting to note that Prp16-fSnap-tagged extracts show no splicing defect relative to wild type extracts. This is somewhat surprising given extensive sequence and presumably structural homology between Prp22 and Prp16. This could possibly reflect differences in accessibility of their binding sites on spliceosome.

In any event, the second step kinetic effects did not preclude use of Prp22-fSnap-tag in CoSMoS experiments since the associated splicing activity was within the original variability used for splicing CoSMoS experiments (Anderson and Hoskins, 2014; Hoskins et al., 2011). Indeed, it is possible that the tagged strain increases the rate of some off-pathway event, such as a quality control decision point. Thus the tagged strain may have made it possible for use
to directly observe both on and off pathways events in one CoSMoS experiment. In going forward, however, I would repeat experiments with a longer linker between Prp22 and fSnap-tag. This has been useful in restoring activity of difficult proteins (personal communication Aaron Hoskins, Andrew Franck).

3.3.2 Prp22 binding stoichiometry

Using a CoSMoS 3-color experiment with dual labeled Prp22 extracts, I was able to get a glimpse of Prp22’s binding stoichiometry. This was previously unknown and not readily determined in ensemble experiments. Proteomics experiments require stable complexes, and indeed rely on depletion and/or mutant DEAH-box proteins such as Prp22 to accumulate distinct spliceosome complexes (Lardelli et al., 2010). My CoSMoS experiments suggest that only one Prp22 protein binds the spliceosome at a time, which hints at the mechanism of Prp22-catalyzed mRNA release. It does not appear to be concerted (i.e., multiple Prp22 proteins required), yet multiple binding events of different Prp22 molecules can occur, sometimes within seconds of each other.

The approach I used is straightforward in that it only requires labeling of the same protein with 2 different colors and looking for the presence or absence of overlaps. The caveats of this approach in this case include non-ideal ratios of green to red dyes, false-negatives (unlabeled or dark proteins) and possible non-specific interactions from the dyes used. In particular, the red dyes used for Snap-tag labeling do not typically perform well in CoSMoS experiments due to non-specific surface binding and/or high background. Andrew Franck and Joerg
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Braun have recently employed Snap-tag beads to sponge up excess amounts of dye from labeled extracts, which substantially reduces the background due to unreacted dye. This approach should be tried in the future with Prp22-fSnap. Nonetheless this was one of the first times such a CoSMoS experiment has been tried and highlights the power and simplicity of the approach. Recently a similar approach was used to observe via CoSMoS-FRET the dimer formation of Mcm2-7, a helicase involved in DNA replication (Ticau et al., 2015).

3.3.3 Prp22 dynamics relative to NTC (spliceosome activation)

The NTC is a critical spliceosome component whose stable association after tri-snRNP binding and U1 and U4 snRNP release coincides with spliceosome activation. Recent structural work belies the importance of the NTC in providing an overall framework for the spliceosome core, and hints at a possible role as a docking platform for other splicing proteins (Yan et al., 2015).

CoSMoS data not only allows direct observation of order-of-assembly events, but also can reveal the kinetic details in the timing of these events. In this case, I asked how long after NTC binding does it take for Prp22 to arrive? The results in this chapter show that in general Prp22 binds after NTC. This was expected in that Prp22’s action during the second step is known to follow spliceosome activation. That said, Prp22 is a RNA-binding protein and could associate with the pre-mRNA and/or one or more snRNAs irrespective of timing of splicing events. Another possibility is Prp22 could interact weakly or short-lived well before its action is required. The Lührmann group recently claimed
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such early interactions for Prp16 and Slu7, but they did not monitor Prp22 (Ohrt et al., 2013). My data indicate these possibilities are unlikely in that the vast majority (>90%) of Prp22 events occur after NTC arrival.

By examining the time difference in overlapped Prp22 and NTC events, I could ask how long after NTC binding does it take for Prp22 to arrive? I observe that Prp22 tends to appear within minutes after NTC arrival. While this is not a novel observation per se, it is interesting in that it places Prp22 binding dynamics relative to spliceosome assembly events. Specifically, since we use NTC as a reference for spliceosome activation, then clearly Prp22 associates after activation.

Following activation, the spliceosome goes through the chemistry of splicing and intron release within a few minutes (Hoskins et al., 2011). This time delay was also observed in CoSMoS-FRET experiments wherein the dynamics of the pre-mRNA were timed relative to NTC arrival (Crawford et al., 2013). In that work, pre-mRNA dynamics were measured by FRET between the 5'SS and the branch point relative to NTC binding. This revealed a transition from a low FRET state to a toggling between mid and high states after NTC arrival. This toggling of FRET states is coincident with spliceosome remodeling events and the chemical steps of splicing. It would be quite interesting to use Danny Crawford’s CoSMoS-FRET approach with Prp22 (or Prp16) labeled extracts. In this manner, the Prp22 binding activity I have observed could be timed relative to both pre-mRNA conformational dynamics and intron fate.
Prp22 has long been known to promote release of spliced mRNA from the spliceosome (Company et al., 1991). Prp22 also presumably uses a similar activity to disassemble spliceosomes caught in off-pathway reactions. Yet the mechanism of mRNA release or even what that entails are not well established. The Lührmann lab has examined disassembly pathways by proteomics of lariat-containing spliceosomes that were reconstituted by adding recombinant Prp22 and Prp43 proteins (Fourmann et al., 2013). Recently Weijun Chen has performed deep sequencing of such complexes in *S. pombe*, but these studies rely on stable complexes remaining after Prp22 action (Chen et al., 2014). Possible disassembly models include the orders of dissociation (NTC->Prp22, Prp22->NTC, or NTC=Prp22) which are not necessarily mutually exclusive. My data shows evidence for all 3 possibilities, with the majority of events being Prp22 disappearance first. About half of the time, another molecule of Prp22 rebinds the NTC, indicating that Prp22 is much more dynamic than the larger complex. This high dynamics of Prp22 relative to NTC is consistent with the model that Prp22 functions as a proof-reader to help resolve spliceosomes that are somehow kinetically stalled in an inactive conformation (Chen et al., 2012; Mayas et al., 2010; Semlow and Staley, 2012).

Another intriguing feature of the Prp22 and NTC ordering is the extent of their simultaneous disappearance. Although it is impossible without proper photobleaching controls to definitively distinguish between dissociation and photobleaching of a single fluorophore labeled protein, a strong argument can be
made about the extreme unlikelihood for simultaneous photobleaching of 3 different fluorophores labeled on 3 different proteins at the level observed in my experiments. Given that these simultaneous disappearances are most likely real, what then do they represent? It is tantalizing to suggest these events reflect successful splicing and dissociation of the mRNA from the spliceosome. Another possibility is an off-pathway reaction proofread by Prp22. Indeed, given the splicing defect of the Prp22-Snap-tagged WCE, there may well be a combination of both on- and off-pathway events.

By parsing analysis of Prp22 binding events into those that occur either in the absence of NTC or overlap with NTC binding, I was able to show that long-lived Prp22 events (~70 sec mean dwell time) only occur in the presence of NTC. That Prp22 binding events overlapping the NTC all had the same mean dwell time hints at how Prp22 works. Consistent with the above stoichiometry studies, the fluorescence intensity traces indicated that only one Prp22 molecule can bind simultaneously. Yet, sometimes there are multiple rounds of Prp22 binding to NTC-containing spliceosomes. Because all Prp22 dwells overlapping NTC were the same regardless of whether they were the first or later binding events, it appears that the binding site for Prp22 is created in a single step. That is, unlike the case for NTC binding where there is evidence for an initial weak binding site preceding formation of a strong binding site (Hoskins et al., eLife, under review) there are no discernible dwell time differences between first, middle or last Prp22 binding events. I observed no short lived binding attempts at the beginning of
any Prp22-NTC overlaps, meaning that Prp22 binding is not in any way initially restricted.

Although I observed no differences in dwell times, there was a difference in wait times. When multiple Prp22 binding events occurred per NTC event, the mean wait time for the last Prp22 event was much shorter than the first (33 vs 179 seconds). Following activation (i.e., binding of NTC), Prp22 binds within minutes and is thought to follow the remodeling activity of Prp16 during the transition from the 1st to second step in which the proteins Cwc25 and Yju2 are displaced (Tseng et al., 2011). Perhaps Cwc25 and Yju2 occlude the binding site(s) of the second step factors Prp22, Slu7 and Prp18. This would also be consistent with the 2-state model of spliceosome dynamics in which the spliceosome is in equilibrium between 2 conformational states that favor either the first or second step of splicing (Konarska et al., 2006). Perhaps the longer initial wait time for Prp22 is a result of the spliceosome being driven from activation to a first step conformation. It would be interesting to repeat these Prp22 experiments with extracts also containing Prp16 (favors the first step conformation) or with Prp8 mutant suppressors that favor one step or the other (Abelson, 2013; Liu et al., 2007).

In kinetic proofreading, there have been 2 models as to how the proofreader works: as a timer or a sensor (Semlow and Staley, 2012). These models are not necessarily mutually exclusive, but evaluation of these models has not been possible using ensemble methods. This is a case where directly
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watching the dynamics and therefore having access to the underlying kinetics is a huge advantage to single molecule methods (Qian, 2008). My observation of no significant differences in the wait or dwell times for Prp22 binding to the spliceosome is more consistent with Prp22 acting as a timer than a sensor. Convincing demonstration of this, however, awaits additional experimental data wherein more events can be analyzed. It will also be quite interesting to repeat these Prp22 experiments using a mixture of wild type (with labeled intron) and 3′SS mutant pre-mRNAs on the same surface in an extract in which the NTC or Prp43 (necessary for spliceosome disassembly) is also labeled.

3.3.4 Prp22 and NTC departure relative to intron release (splicing)

I also attempted to frame these Prp22 and NTC events in the context of splicing by performing a 3-color CoSMoS experiment using the RPS30A pre-mRNA with a labeled intron. Although the number of pre-mRNA molecules for which I could confidently distinguish intron departure from photobleaching was too small to yield reliable statistics, it was intriguing to examine the intron fate for this small subset (see below). That both spliceosome assembly and splicing occurred on this labeled pre-mRNA was confirmed by the ATP dependence of Prp22 and NTC binding (spliceosome assembly/activation) and intron disappearance (splicing and intron departure). This rules out nuclease activity as a primary reason for intron disappearance, since labeled pre-mRNA molecules were present throughout the experiment for Prp22 and NTC to interact
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with. The data also confirmed that Prp22 and NTC ordering and binding behaviors on RPS30A pre-mRNA are in general similar to what I observed with RP51A pre-mRNA.

After careful analysis individual traces, I identified 15 pre-mRNA molecules for which disappearance of the intron signal most likely resulted from splicing. For 3 of these pre-mRNAs, the final Prp22 and NTC departures occurred in the interval between the final intron observation and the first time point when the intron was no longer visible. These events most likely represent successful splicing and intron departure. In all 3 cases, Prp22 and NTC disappeared simultaneously, consistent with the current model that the upon spliced exon release by Prp22, the lariat intron product departs as ILS complex containing the NTC and likely also Prp22. Lack of Prp22 detection in the S. pombe ILS (Chen et al., 2014) suggests that Prp22’s association with ILS is transient.

The 9 cases where loss of intron signal occurred after final Prp22 and NTC departure are intriguing in that they may represent spliceosome discard. While it is well established that Prp22 is a proofreader of the second step (Mayas et al., 2006), how it achieves this and what the discard pathway entails is unknown. For 3 pre-mRNA molecules wherein the last Prp22 and NTC disappearances occurred prior to loss of intron fluorescence, Prp22 and NTC disappeared simultaneously. This suggests that one outcome of a Prp22-dependent discard pathway is loss of either NTC or the entire spliceosome. In
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future experiments it will be interesting to explore in greater detail these splicing and discard pathways. Rather than rely on loss of a labeled intron, perhaps direct detection of the spliced mRNA by an exon-junction targeting probe (e.g., fluorescently labeled gamma-PNA (Dragulescu-Andrasi et al., 2006)) would be more straight-forward.

3.3.5 Perspective

In the work described here, I demonstrated that CoSMoS can be used to observe the comings and goings and binding stoichiometry of the splicing factor, Prp22, on individual pre-mRNA molecules. This extends CoSMoS analysis of spliceosome dynamics to protein molecules that bind individually rather than as part of a larger complex. Further I demonstrated that Prp22 binding is dynamic and occurs after spliceosome activation (i.e., NTC arrival). Finally, my data have provided a first glimpse into the ordering of a likely Prp22-dependent spliceosome discard pathway. My experiments thus lay the groundwork for future researchers to dissect the late stages of spliceosome action, disassembly and quality control using single molecule methods.
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3.4 Materials and Methods

3.4.1 Preparation of whole cell extracts

Yeast extracts were prepared from various strains with Prp22-fSnap and/or NTC-double-DHFR (Cef1-DHFR, Ntc90-DHFR) as described previously (Hoskins et al., 2011) and in section 2.3.

3.4.2 Preparation of fluorescent pre-mRNAs

The RP51A pre-mRNA was biotinylated and labeled with a single Alexa488 fluorophore as described in section 2.1.

The RPS30A pre-mRNA with a labeled intron was designed and prepared as before (Shcherbakova et al., 2013). However, a biotin handle oligo was ligated to the 3’ end of the pre-mRNA in place of the Klenow extension used in the reference.

3.4.3 Construction of flow chambers

The microscope slides and flow chambers used in these CoSMoS experiments were prepared as described in section 2.5.

3.4.4 CoSMoS TIRF microscopy

Splicing reactions and fluorescent Prp22 and NTC images were recorded using a multi-wavelength single molecule fluorescence microscope that has been described previously (Friedman et al., 2006) and GLIMPSE software (https://github.com/gelles-brandeis/Glimpse). The single molecule splicing assays were assembled as described in section 2.7. The acquisition settings used
were similar to previous CoSMoS splicing experiments (Hoskins et al., 2011; Shcherbakova et al., 2013).

3.4.5 Analysis of CoSMoS data

The recorded images from the CoSMoS experiments were analyzed using custom Matlab software created and provided by Larry Friedman (https://github.com/gelles-brandeis/CoSMoS_Analysis) and has been described previously (Friedman and Gelles, 2015).
4.1 Development of CoSMoS methods for studying splicing

The CoSMoS technique was established by Larry Friedman and Jeff Gelles nearly 9 years ago and has fundamentally advanced our understanding of not only spliceosome assembly and pre-mRNA splicing, but a wide range of biological processes. To achieve this understanding required an incredible amount of methods development, troubleshooting and extensive combination of various disciplines including yeast genetics, fluorescently labeling of proteins and RNA, surface chemistry, TIRF microscopy and image analysis. This would not be possible without the partnership between the Moore and Gelles labs, and collaboration with New England Biolabs and Virginia Cornish for orthogonal protein labeling technology.

My thesis research using CoSMoS started with a lab rotation in the Moore lab under Aaron Hoskins. I was tasked with finding a way to specifically label proteins in yeast extracts to be later be used in CoSMoS splicing experiments. We quickly arrived at using Snap-tag labeling technology as one of the few methods that was compatible with extracts and in vitro splicing conditions. The problem however was removal of excess dye from the labeling reaction – even with a small excitation volume, the TIRF microscope used for CoSMoS does not tolerate high backgrounds. I recalled during my rotation in the Jacobson lab the yeast extracts used in ribosome toeprinting assays were prepared via gel
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filtration chromatography. Traditionally splicing extracts are dialyzed, but this does not sufficiently remove the free Snap-tag dye. Using my love of chromatography, I was able to develop a size exclusion chromatography method that separated the bulk of free dye from the extract. Critically, this did not fractionate the extract or alter the associated splicing activity. This method of dye removal from labeled extracts became instrumental to the further development and success of CoSMoS splicing experiments, and is the salient feature common to all of the CoSMoS splicing publications to date. Indeed, without the specificity of Snap-tag labeling and the low background afforded from the dye removal method, there would not be many spots to see and count during a splicing reaction.

4.1.1 Future development of CoSMoS

The power of the CoSMoS method for studying the dynamics of biomolecules as they come and go lies in its simplicity and versatility. Interested researchers need only have one labeled biomolecule tethered to the slide surface, from which they (hopefully) observe the colocalization of another labeled biomolecule. But this versatility is betrayed by the amount work and care required for acquisition and analysis of the images. Both processes have been quite labor intensive, and in some aspects there is (or should be) no avoiding it. Nonetheless, the software tools employed to acquire and analyze CoSMoS have improved significantly in the past few years. My first CoSMoS experiments consisting of <100 RNAs to analyze required over 1 day of computer processing,
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followed by weeks of manual analysis. Currently, thanks to improvements made by Larry Friedman in the Matlab code employed (Friedman and Gelles, 2015), I can process ~500 RNAs in less than a half hour. Sadly, the subsequent analysis, while semi-automated, requires careful manual supervision and still takes weeks before I know if the experiment truly worked or not. This will not change until new methods are used for CoSMoS image analysis (Smith et al., 2010), which Andrew Franck and Carlas Smith are attempting to establish. If successful, these new methods potentially allow the analysis of a CoSMoS experiment in near real-time with an accuracy in spot detection currently not possible. Finally, it would be extremely constructive if the community of CoSMoS researchers adopted a storage of annotated and standardized data/methods that could later be utilized as a resource. With adaption of electronic notebooks, this should no longer be an issue and will help standardize CoSMoS from a boutique method.

4.2 The dynamics of Prp22 during splicing

After helping develop the CoSMoS toolkit, my thesis research switched to applying those tools to the study of trans-acting helicases involved in promoting the fidelity and conformational rearrangements associated with splicing. I was quite interested in watching the dynamics of Prp22 during splicing and was inspired by the work from Jon Staley’s lab demonstrated Prp22 as a proofreader of the second step of splicing (Mayas et al., 2006). The actual mechanism of
Prp22 as proofreader and the nature of the discard step were open questions that could be answered by my CoSMoS experiments. By definition, anything I saw would be novel, yet it was not so. Plagued by variability in yeast extract preparations, microscope slide stickiness, TIRF microscope adjustments, etc. I could not distinguish between Prp22’s real and dark control events, let alone say anything about proofreading. It wasn’t until I saw the clarity afforded Danny Crawford’s CoSMoS-FRET experiments by combining a labeled spliceosomal subcomplex that I decided to monitor Prp22 relative to spliceosome assembly and splicing itself.

By using a yeast strain in which Prp22 and the NTC were respectively fSnap and DHFR tagged, I could prepare extracts for watching Prp22 and NTC binding events during splicing. I was initially disappointed to merely confirm the expected ordering of NTC appearing first, later followed by Prp22. Moreover, my experiments were still plagued by nonspecific dye accumulation on the slide surface – sometimes it was the green Snap-tag dye (Prp22), sometimes the red DHFR-Cy5-TMP (NTC). I finally realized I could actually ignore the nonspecific background (provided it was not dominating the experiment) and focus on events in which Prp22 and NTC colocalized on the same RNA at the same time. In effect, I was using NTC binding events as a filter for selecting possibly interesting Prp22 events.

To aid in my analysis, Larry Friedman kindly provided me with a matlab script that detected such overlaps. I have added to and refined the script to the
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point where such overlap analysis for a 3-color CoSMoS experiment is routine. Analysis of the Prp22/NTC overlapping events provided a detailed look at Prp22 dynamics during splicing that have been heretofore unknown. I find that the apparent ordering shows a 2-3 min delay from NTC binding before the first Prp22 binding event in that overlap. After that initial Prp22 binding event, the wait times decrease to ~1 min. The longer initial wait time suggests Prp22 is somehow restricted from binding to the spliceosome, presumably until after the first step. This delay is similar to the one Danny Crawford saw for onset of toggling of a mid to high FRET state after NTC arrival (Crawford et al., 2013). Again, it would be tantalizing but not at this time feasible to combine labeled Prp22 extracts with Danny’s FRET pre-mRNA to see where Prp22 events would be in the sea of FRET dynamics.

Of especial interest to me is the simultaneous disappearance of Prp22 and NTC signal. This occurs fairly frequently (30% of Prp2/NTC overlaps) and possibly hints at either a spliced mRNA release or discard step. I would love to follow these experiments up with different extracts in which Prp22 would be monitored with other labeled snRNPs (U5 and U2) and observe their possible initial disassembly events.

I tried to follow up this observation by also monitoring the fate of the intron using the labeled RPS30A intron. Inna Shcherbakova first designed and used this pre-mRNA to monitor splicing (Shcherbakova et al., 2013). The long intron of RPS30A allows the use of one color to monitor its fate. Unfortunately in my
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case the labeling of the intron was not extensive enough to distinguish intron release from photobleaching. Especially disappointing was the lack of difference in intron signal lifetime in the +/- ATP experiments. Yet both Prp22 and NTC provided RNA and ATP dependent events, which is evidence the experiment itself worked. Nonetheless it is quite intriguing to see cases in which the intron loss signal while Prp22 and NTC were present (possible splicing) and when Prp22 and NTC departed simultaneously but the intron clearly remained.

Going forward I would like to prepare a brighter splicing reporter to be used in follow experiments. I would of course also try reducing the second step splicing defect associated with Prp22-fSnap by using a longer linker and/or using more concentrated extracts that Andrew Franck and I have been developing. Finally, I would also repeat the Prp22/NTC experiments to establish both reproducibility and the extent of photobleaching. As such my current dataset serves as a preliminary data that illustrates the possibilities of monitoring a trans-acting helicase during splicing.

4.2.1 Future CoSMoS experiments with Prp22 and other splicing ATPases

The identity of the splicing ATPases are prominently linked with splicing fidelity and the theory of kinetic proofreading. Sean Burgess and Christine Guthrie first beautifully demonstrated the ability of Prp16 helicase mutants to allow for stabilization of mutant branch point containing lariat intermediates in yeast cells (Burgess and Guthrie, 1993). From these observations came idea of the splicing helicases acting as a “proofreading clock”. Later Beate Schwer
demonstrated the ability to immunodeplete Prp16 from yeast extracts, then restore its function by adding back recombinant Prp16 (Schwer and Guthrie, 1991). This technique has been adopted and used nearly ad nauseam by Jon Staley and Soo Chen-Cheng in a series of increasingly convoluted experiments to dissect and demonstrate the functional and fidelity roles of Prp16 and other DEAH-box proteins (Koodathingal et al., 2010; Tseng et al., 2011).

From those complicated experiments, David Horowitz has extracted an interesting theory or flavor of proofreading in which the helicases don’t act as proofreaders per se (Horowitz, 2011). Rather, and in the case of Prp16, it is the protein Cwc25 that performs the fidelity check of the branch point and Prp16 acts as an ‘expediter’ moving the splicing reaction forward. Upon examination of the spliceosome assembly and splicing pathway, it becomes apparent this model of pairing a sensor protein with an ATPase could be applied to nearly all of the transitions these helicases govern (refer to Figure 1.4). It would be interesting to evaluate this model using CoSMoS splicing experiments in which different splicing proteins are orthogonally tagged and observed against wild-type and mutant pre-mRNAs. To this end, I already have the different combinations of yeast strains prepared (e.g. Prp22/Slu7, Prp16/Cwc25, Prp2/Spp2), which could be combined with a labeled intron containing pre-mRNA to watch the comings and goings of splicing (Figure 4.1)
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Figure 4.1 Exploring the DEAH-box proteins using CoSMoS. Scheme illustrating the DEAH-box proteins Prp2, Prp16, Prp22 and Prp43 and the key steps or transitions they promote. The colors are meant to show the possible combinations for future CoSMoS splicing experiments through orthogonal labeling and pairing with either labeled spliceosome components or a pre-mRNA with a labeled intron.
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4.3 Final thoughts

I would like to close with a quote from Paul de Kruif’s *Microbe Hunters* attributed to Antonie van Leeuwenhoek (de Kruif, 1962):

*People who look for the first time through a microscope say now I see this and then I see that – and even a skilled observer can be fooled. On these observations I have spent more time than many will believe, but I have done them with joy, and I have taken no notice of those who have said why take so much trouble and what good is it? – but I do not write for such people but only for the philosophical!*

In his near breathless style of prose, Paul de Kruif paints a picture of Leeuwenhoek as a careful experimenter, constantly searching and reevaluating what he was seeing, always questioning. As the father of Microbiology and an accomplished microscope builder, Leeuwenhoek saw something novel every time he peered into the then unknown microscopic world. Leeuwenhoek’s balancing curiosity and caution in exploring new things is a mindful lesson for those today using single molecule microscopy methods I think. In helping establish the tools and methods used for CoSMoS splicing experiments I am quite mindful of the need to practice care and restraint in collecting and analyzing data. Yet I have also experienced the allure and amazement of simply counting spots and look forward to what those spots can reveal.


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