Final Report

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Biochemical and Physiological Characterization: Development & Apply Optical Methods for Characterizing Protein-Protein Interactions in MR-1 (Objective 3)

Project ID: 0007461

Objectives

1. Develop novel site-specific protein labeling chemistries for assaying protein-protein interactions in MR-1.
2. Development of a novel optical acquisition and data analysis method for characterizing protein-protein interactions in MR-1 model systems.

Progress and accomplishments

Our work on analyzing protein-protein interactions in MR-1 is divided in four areas:

- (1) expression and labeling of MR-1 proteins
- (2) general scheme for site-specific fluorescent labeling of expressed proteins
- (3) methodology development for monitoring protein-protein interactions, and
- (4) study of protein-protein interactions in MR-1.

In this final report, we give an account for our advances in all areas.

1. Overexpression, purification and characterization of *Shewanella oneidensis* MR-1 proteins

Since our transition from periplasmic proteins to cytosolic transcription factors, we have been very successful in overexpressing and purifying these proteins. We have received plasmids encoding hexahistidine fusions (His₆-fusion) of MR-1 proteins, ntrC, rpoN (σ^{54}), ihfA (α subunit), ihfB (β subunit), and single cysteine mutations of ihfA^C and ihfB^C from Dr. Margie Romine at Pacific Northwest National Lab (PNNL). In addition to these clones, we have, in collaboration with Dr. Jeanne Perry at the DOE Protein Expression Facility at UCLA, created plasmid clones of MR-1 proteins rpoE (σ^{24}), rpoD (σ^{70}), and rpoH (σ^{32}), as well as, single cysteine mutations of ntrC (ntrC^C) and rpoE (rpoE^C).

We began by attempting to reconstruct the two-component signaling cascade involving NtrC, σ^{54} , and IHF (heterodimer of α and β subunits). Transcriptional activation of σ^{54} is achieved through a protein-protein interaction between a transcription regulator, NtrC, and the σ^{54} -RNAP (E σ^{54}) holoenzyme complex. At some promoters, an additional DNA structural factor, IHF, is required to initiate the DNA looping event to activate transcription[1].

Figure 1. Overexpression and purification of MR-1 proteins. Correct proteins are indicated with arrows. A. NtrC, left expression study, middle western blot of overexpression, and right, native Ni-NTA purification. B. σ^{54} , left expression study, middle western blot of overexpression, and right, denaturing Ni-NTA purification. C. Overexpression of both subunits of IHF α (right) and β (left). D. σ^{24} , left expression study, middle western blot of overexpression, and right, native Ni-NTA purification.

We have identified promoter systems in MR-1 that have the necessary binding sites to reconstruct this signaling cascade. The proteins, NtrC, σ^{54} , and both ihfA and ihfB have all been successfully overexpressed (Fig. 1A-D). NtrC and its single cysteine mutation NtrC^C were both partially soluble under native purification conditions and were purified using Ni-NTA affinity chromatography (Fig. 1A, right). The purification σ^{54} did require a denaturing protocol with a protein refolding procedure, which is not uncommon for sigma factors[2](Fig. 1B, left). This refolding procedure has resulted in folded protein verified by circular dichroism measurements. Both native IHF subunits purify under native conditions, but a large majority of the protein is insoluble. This may indicate that the subunits need to be co-expressed to increase the soluble portions[3]. Co-expression constructs are currently being developed.

Preliminary characterization of these proteins has yielded mixed results. Gel shift characterization of both NtrC proteins has been largely inconclusive, and an early transcriptional test, described in section 4, indicated the protein may not be active. For σ^{54} , early gel shift assays have indicated some compatibility with *E. coli* RNAP core and affinity for known σ^{54}

promoters, and we are currently creating a single cysteine mutation of σ^{54} . For these constructs refining refolding procedures could lead to greater proportions of active proteins, and we are currently optimizing purification protocols for all of these proteins.

In addition to reconstructing the two-component signaling cascade, we also reconstructed a transcription system that does not require transcriptional activators, σ^{24} . We have successfully overexpressed soluble σ^{24} and purified it using Ni-NTA affinity chromatography with high yield, 1.5 mg/L (Fig. 1D). Gel shift characterization of σ^{24} has indicated compatibility with *E. coli* RNAP and MR-1 promoters (results are described in section 4). With these positive indications, we are currently creating a single cysteine mutant of the σ^{24} protein for labeling.

2. Development of an organometallic reagent for site-specific labeling of His₆-fusion proteins

Development of reagents and methods to site-specifically label proteins has been increasingly important to investigate macromolecule interactions. We are developing new method for site-specific fluorescence labeling of proteins. **(NOTE: This part of the work is confidential and will result in a patent application. We ask this information not to be shared with others.)** We are focusing on the hexahistidine tag (His₆-tag) as a unique affinity target for direct, site-specific labeling.

Our method is based on His₆-tag-mediated labeling with Ni²⁺-Fluorochrome conjugate[4]. Kapanidis et. al. reported that their [Ni²⁺:NTA]₂-cyanine fluorochromes were able to target His₆-tag proteins for specific labeling; however, their reagents have intrinsic problems. Dissociation occurs on a short time scale, and the binding does not survive electrophoresis indicating low affinity. Thus, their applications for protein labeling are limited, and not compatible with single molecule analysis, where picomolar (pM) concentrations of labeled proteins are used.

We hypothesized that the two [Ni²⁺:NTA] moieties may not interact tightly with the His₆-tag, and the introduction of more [Ni²⁺:NTA] moieties into fluorochromes might increase the binding affinity. Based on this hypothesis, we synthesized new labeling reagent, [Ni²⁺:NTA]₄-dT-fluorochrome. Using oligonucleotide as a backbone, we have generated chimeric oligo dT containing two dithiol groups and fluorophore. The reagent was further chemically modified to introduce four [Ni²⁺:NTA] and was converted to [Ni²⁺:NTA]₄-dT-fluorochrome.

Our preliminary result, a electrophoresis-based binding assay (Fig. 2), indicates that the new reagent is superior to [Ni²⁺:NTA]₂-cyanine fluorochromes. The label remains attached to the His₆-tag during electrophoresis for more than 3 hours, whereas the old version, [Ni²⁺:NTA]₂-cyanine fluorochrome, failed to show any detectable binding activity (A. Kapanidis and R. Ebright, unpublished). This method is now available to apply to any His₆-tag MR-1 proteins and is most likely suitable for single molecule ALEX analysis (see below).

3. Development and application of optical methods for the analysis of protein-protein interactions in *Shewanella oneidensis* MR-1

A. μ s-Alternating-Laser Excitation (μ s-ALEX) spectroscopy

Formerly, to study structure and dynamics, we used single laser excitation single molecule Förster resonance energy transfer (smFRET), while using this technique it became apparent to us that smFRET (a method that studies the non-radiative energy transfer process from a fluorescent donor, D, and a fluorescent acceptor, A) was not a *general* platform for quantitative analysis of structure and interactions, it does not probe *directly* the presence and the properties of the acceptor. We thus introduced alternating-laser excitation (μ s-ALEX) spectroscopy[5. NKL et al., in press] a method that rapidly (μ s timescale) switches between a laser exciting a FRET donor and a laser exciting a FRET acceptor to obtain both donor-excitation– and acceptor-excitation–based observables for *each single molecule* (Fig.3A &B). μ s-ALEX recovers distinct emission signatures (Fig. 3C) by calculating two fluorescence ratios: the FRET efficiency *E*, which reports on donor-acceptor distance[6], and the distance-independent ratio *S*, which reports on donor-acceptor stoichiometry. These orthogonal ratios allow virtual molecule sorting (Fig. 3C), thermodynamic and kinetic analysis of interactions, identification of interaction stoichiometry, and study of local environment. When applied to diffusing

Figure 2. Organometallic reagent for hexahistidine labeling. A. [Ni²⁺:NTA]₄-dT-fluorochrome. Four molecules of Ni²⁺:NTA are attached to DNA backbone structure. B. Nondenaturing electrophoresis. NtrC with C-terminal his-tag was reacted with [NTA]₄-dT-fluorochrome(lane 1) and [Ni²⁺:NTA]₄-dT-fluorochrome (lane 2).

molecules, μ s-ALEX enables homogeneous, “mix-and-read” assays, in which optical readouts report *simultaneously* on the association and conformation status of single molecules.

In our previous progress report, we described early characterization of various labeled DNA model systems with μ s-ALEX, and expanded its application to protein-DNA interactions by using the model system of catabolite activator protein (CAP) with DNA. All of these experiments validated the ability of μ s-ALEX for simultaneous, independent monitoring of distances and stoichiometries, and its use to study equilibrium binding and determine kinetic rate constants[5]. With the potential of μ s-ALEX clearly established, we continued to expand and refine the information μ s-ALEX provides about interacting partners by: i) determining accurate FRET efficiencies for interacting pairs, ii) expanding μ s-ALEX to three lasers- three colors, and iii) applying of μ s-ALEX to the analysis of a complex protein-DNA interaction.

i. Accurate- E and single-base pair resolution.

Measurements of accurate distances would further enhance the analysis of structures and interactions. An intermediate step in the derivation of accurate distances requires the extraction of accurate FRET efficiencies. Using μ s-ALEX to sort species based on donor-acceptor distance and stoichiometry, we have determined the correction factors necessary for determining accurate-FRET efficiencies(NKL et al., in press). These factors account for crosstalk terms contaminating the FRET-induced acceptor-emission (factors d and l ; Fig. 4A-B), and for differences in the detection efficiency and quantum yield of the fluorophores (factor γ ; Fig. 4A-B), all of which are independent of instrument-specific factors. Using DNA fragments, we showed that ALEX-based intramolecular DNA distances agree with predictions from a cylindrical model of DNA helix [7](Fig. 4C). Additionally, we were able to distinguish clearly between differences in D-A separation as small as a single basepair (Fig. 4D).

We also examined protein-DNA interaction distances using RNA-polymerase(RNAP) -DNA complexes, and we have preliminary data that detect single-basepair translocation of RNAP on DNA by populations of elongation complexes (A. Kapanidis and S. Weiss, unpublished). The high spatial resolution of μ s-ALEX will help dissect the mechanism of RNAP translocation during initiation and elongation, and its coordination with the enzymatic step. These measurements yielded distances in agreement with ensemble-FRET measurements, and with models based on systematic-FRET and X-ray crystallography[8-10]. Therefore, μ s-ALEX complements structural analysis of biomolecules, especially for species intractable by conventional structural biology due to heterogeneity, limited stability, large size, flexible domains, or transient nature.

ii. 3-color ALEX.

Since biological mechanisms couple multiple association/dissociation events with series of conformational changes, simultaneous observation of *multiple* interaction stoichiometries and *multiple* distances is clearly desirable. We developed a general method for simultaneously measuring multiple interaction stoichiometries and up to three interprobe distances within single molecules. This is performed by using three alternating lasers (488-, 543-, and 638-nm light) (Fig. 5C) to implement μ s-ALEX in solutions containing molecules labeled with three spectrally distinct fluorophores (Alexa488, TMR, and Alexa647); we defined this method as 3-color μ s-ALEX (3c-ALEX; N. Lee, A. Kapanidis and S. Weiss, in preparation).

Using 3c-ALEX, we sorted and identified triply labeled diffusing DNA fragments present in heterogeneous mixtures (Fig. 5A-B), and measured *simultaneously* three distances within them. 3c-ALEX could clearly distinguish between three triply labeled DNA fragments with distinct interprobe distances (Fig. 5D); sorting and identification of triply labeled species was based on stoichiometry, whereas distinguishing between triply labeled DNA was based on interprobe distances. 3c-

Figure 3. A. ALEX instrumentation. **B.** Observable for a diffusing molecule with a D and an A but no FRET; acceptor presence is detected through direct A-excitation for a fraction of acquisition time. **C.** 2-D histograms of E (FRET efficiency) and S (stoichiometry) sort molecules according to stoichiometry and interprobe distance. D-only species appear with low E and high S ; A-only species appear with low S ; all D-A species displayed intermediate S and distance-dependent E .

Figure 4. A. Schematic of species with different FRET and stoichiometry needed to calculate correction factors l , γ , and d and obtain accurate FRET. **B.** Sample data of species in panel A. **C.** Single-molecule FRET as a function with D-A pair separation (red circles) compared with a cylindrical model of DNA (black line); ALEX-based distances fit better than ensemble-based distances (open circles). **D.** ALEX distinguishes between dsDNA fragments of 15, 16, and 17 bp D-A separation (main species with intermediate FRET), achieving single-basepair resolution on the single-molecule level.

ALEX should be instrumental for quantitative analysis of static and dynamic biomolecular structure as well as of biomolecular interactions. Extension to a 4-color implementation (and, in principle, *up to six distances within a single molecule*) is possible by taking advantage of a spectral window between TMR and Alexa647 that can be used to introduce a fourth fluorophore (e.g., Alexa594). Finally, 3c- or 4c-ALEX will probe interactions between 3 or 4 molecules (each labeled with a spectrally distinct fluorophore) by checking the coincidence of fluorophores in the resulting complexes.

iii. Application to a complex protein-DNA interaction

We have used our methods to study a transcription mechanism rich with transient intermediates, release of σ^{70} upon promoter escape. Textbook versions of transcription postulated obligatory release of σ^{70} upon formation of the first stable elongation complex, formed upon synthesis of a 9-11 nt nascent RNA [11-15]. However, early methods for quantifying σ^{70} release included separation steps that translated a *decreased affinity* of σ^{70} for elongation complexes into *release* of σ^{70} from elongation complexes. There is growing evidence of the presence of σ^{70} in elongation and has led to new proposals about regulatory roles of σ in elongation [16-18]. However, the extent of σ^{70} retention in early and mature elongation complexes, the point(s) where release occurs, and the determinants of the extent and point of release are still unclear, in part due to shortcomings of the available methodology for measuring σ^{70} release.

By combining μ s-ALEX with two complementary experiments used in ensemble assay: leading-edge-FRET (LE-FRET), where FRET probes are introduced at the leading edge of RNAP and the downstream end of DNA, and downstream translocation of RNAP is monitored as a FRET increase (Fig. 6A); and trailing-edge-FRET (TE-FRET), where FRET probes are introduced at the trailing edge of RNAP and the upstream end of DNA, and downstream translocation of RNAP is monitored as a FRET increase (Fig. 6B); we have measured σ^{70} retention at various points along elongation (A. Kapanidis, E. Margeat, J. Mukhopadhyay, R. Ebright and S. Weiss, in preparation). From a single sample without additional controls, μ s-ALEX determines: (i) the fraction of active open complexes; (ii) the fraction of stalled elongation complexes that can resume transcription; and (iii) the fraction of transcription complexes that retain σ^{70} (Fig. 6C & D).

We showed that $\geq 80\%$ of elongation complexes containing 11 nt of RNA retains σ^{70} , and that most of the elongation complexes retain σ^{70} after synthesizing 14-nt of RNA (Fig. 6E). The translocation activity was $>80\%$; the ability of the elongation complexes to resume elongation upon addition of the full set of nucleotides was $\sim 80\%$. A large fraction (25-55%) of mature elongation complexes (containing 50 nt of RNA) retained σ^{70} . Our results support the proposal for non-obligatory σ^{70} release [16, 19], and raise the intriguing possibility of modulation of σ^{70} release by modulating σ^{70} -RNAP interactions in elongation.

The μ s-ALEX methodology represents a marked improvement compared to the original smFRET, since the additional resolution gained by the new observables removes the photophysically-induced "Donor-only" (D-only) peak [6], eliminates contributions from the buffer, and allows the evaluation of binding stoichiometries, complex composition, and effects of local environment without FRET constraints. The only limitation to the μ s-ALEX-FAMS technology is its restriction to low concentration samples (tens of picomolar concentration up to one nanomolar), due to the requirements

Figure 5. A. 3c-ALEX generates 3 2-D probe-stoichiometry histograms. Shown are expected positions of all species. Triply-labeled species (B-G-R, yellow) is in the center. B. Data for triply-labeled unpurified dsDNA. C. 3c-ALEX instrumentation. D. Set of 3 triply-labeled DNA fragments analyzed using 3c-ALEX. ALEX-based distances, black; cylindrical model distances, grey.

Figure 6. Single-molecule analysis of σ release. A. LE-FRET. Release of σ^{70} from the transcription apparatus results in dissociation of a low- E D-A RNAP-DNA complex (left) to free σ^{70} (D-only) and a σ^{70} -free RNAP-DNA complex (A-only). Retention of σ^{70} converts a low- E , D-A species to a high- E , D-A species where σ^{70} and RNAP-DNA complex remain colocalized. B. TE-FRET. Release of σ^{70} results in dissociation of a high- E , D-A RNAP-DNA complex (left panel) to free σ^{70} and a σ^{70} -free RNAP-DNA complex. Retention of σ^{70} converts a high- E , bound species to a low- E , D-A species where σ^{70} and RNAP-DNA complex remain colocalized. C, D. σ^{70} is retained in the first stable elongation complex: E^* -S histograms for open, elongation, and chased complexes studied by LE-FRET (panel C) and TE-FRET (panel D). The D-A and A-only species are separated on the S histograms; the E^* histogram is shown only for the bound species (molecules $0.5 < S < 0.8$, in solid rectangle). E. Retention and stability of σ^{70} in the first stable elongation complex.

of burst analysis, and high affinity interactions ($K_d < 1\text{nM}$)[20]. This limitation poses a challenge for analyzing weaker, transient protein-protein interactions[21].

B. ns-ALEX

Figure 7. A-B. Schematic of burst detection, and lifetime determination by time-stamped photon arrivals. C. Typical burst histogram for multiple species in solution. D-G. Fluorescence intensity decay data of different species in solution from panel C.

and acceptor fluorescence lifetimes (Fig. 7B). In addition, using linearly polarized excitation and employing four detection channels to divide emission by polarization as well as D and A spectra, time-resolved anisotropy and FRET measurements are obtained *simultaneously*.

With these additionally parameters, ns-ALEX measures accurate FRET efficiencies without detection corrections and evaluates the rotational freedom of fluorophores. Molecules with different distance separation between labeling positions are distinguished as “static” species with different values of E , and fluorescence decay data recovers distance distributions for individual species even when the fluctuations occur on time scales faster than the single-molecule burst (diffusion) durations. Single-molecule bursts are still sorted into different species based on the two-dimensional E - S burst histogram. In addition, the histogram can be divided more accurately into specific ranges to give fluorescence lifetimes of subpopulations (Fig. 7C).

For each of the four detection channels, fluorescence lifetime histograms are formed using all of the photons from all of the bursts of each species (Fig. 7D-G). In figures 7D-G, the signatures of subpopulations with D -only, A -only, and high- and low-FRET are clearly distinguished. Since there are two lifetime decays in the A channels arising from D_{exc} and A_{exc} , there are a total of six lifetime decays for all channels. All six fluorescence lifetime decays are globally fitted to provide information about the selected subpopulation.

Figure 8. Left. FCS D and A autocorrelation and D-A cross-correlation. Right. Correlation functions for D and A autocorrelation and D-A cross-correlation. E is the FRET efficiency, and k is the brightness ratio between green and red fluorophores. Concentrations of free species: D -only (Y_D) and A -only (Y_A), and bound species D - A pairs (Y_{DA}) are determined using the correlation function.

improvement for interaction studies by FCS. By doing the leakage-excluded cross-correlation analysis, we are able to recover the non-correlating feature between two singly-labeled molecules which conventional cross-correlation fails to show due to leakage effects. By carefully applying fitting routines for the two-color cross-correlation curves, we are able to extract accurate occupancies for different species (bound/ free) inside the excitation volume (at higher, $\sim\text{nM}$ concentrations) and thus probe weaker binding affinities in the solution (Fig. 8)[20].

In the lower affinity ($K_d > 1\text{nM}$) range, dual-color cross-correlation analysis has been applied to macro-molecular interactions, with the occupancies of different species being resolved from cross-correlation fitting routines. The accuracy of these recovered occupancies, however, is skewed in large scale by donor signal “cross-talk” into acceptor detection channels. To incorporate these lower affinity interactions into our methodology while preserving the advantages of μs -ALEX and eliminating the drawbacks of dual-color cross-correlation “crosstalk”, we developed an extension of the ALEX method called nanosecond-ALEX (ns-ALEX; T. Laurence, X. Kong, and S. Weiss, in preparation).

Similar to μs -ALEX, single molecules are detected using the bursts of fluorescence photons they produced while diffusing through the focused laser excitation volume (Fig. 7A) and are sorted by the distinct emission signatures recovered through the rapid laser alternation (Fig. 3C). While μs -ALEX utilizes alternation periods between 25 and 100 μs , for ns-ALEX, two synchronized picosecond-pulsed lasers are interlaced to obtain 14.7 ns alternation periods.

This faster alternation period still achieves ALEX, while also measuring donor and acceptor fluorescence lifetimes (Fig. 7B). In addition, using linearly polarized excitation and employing four detection channels to divide emission by polarization as well as D and A spectra, time-resolved anisotropy and FRET measurements are obtained *simultaneously*. With these additionally parameters, ns-ALEX measures accurate FRET efficiencies without detection corrections and evaluates the rotational freedom of fluorophores. Molecules with different distance separation between labeling positions are distinguished as “static” species with different values of E , and fluorescence decay data recovers distance distributions for individual species even when the fluctuations occur on time scales faster than the single-molecule burst (diffusion) durations. Single-molecule bursts are still sorted into different species based on the two-dimensional E - S burst histogram. In addition, the histogram can be divided more accurately into specific ranges to give fluorescence lifetimes of subpopulations (Fig. 7C). For each of the four detection channels, fluorescence lifetime histograms are formed using all of the photons from all of the bursts of each species (Fig. 7D-G). In figures 7D-G, the signatures of subpopulations with D -only, A -only, and high- and low-FRET are clearly distinguished. Since there are two lifetime decays in the A channels arising from D_{exc} and A_{exc} , there are a total of six lifetime decays for all channels. All six fluorescence lifetime decays are globally fitted to provide information about the selected subpopulation.

(NOTE: This part of the work is confidential and will result in a patent application. We ask this information not to be shared with others.)

The decreased alternation time period, not only extends the photon burst analysis of the ALEX method, but it improves dual-color cross-correlation analysis. By alternating laser excitations with precise timing and categorizing photon streams according to their excitation source, we can exclude the fluorescence leakages from extended fluorophore spectra and perform dual-color fluorescence correlation spectroscopy (FCS) on a doubly-labeled molecule or bound species of two different singly-labeled molecules, which will be a great

Figure 9. Quantification of unlabeled DNA target. **A.** Model of hybridization, two fluorescently-labeled DNA probes hybridize to target DNA. **B.** ns-ALEX-FACS FCS analysis of hybridization, subpopulations are determined and quantified using the correlation function. **C.** Titration curve for concentration determination. **D.** Calibration curve for a series of targets measured this way.

To demonstrate the robust ability of ns-ALEX, we monitored the hybridization of a D-labeled probe and an A-labeled probe to an unlabeled target DNA molecule (Fig. 9). Hybridization was monitored by titration of an increasing concentration of probes against a constant concentration of target. Cross-correlation was used to calculate the concentration of bound species at each point and a titration curve was constructed to calculate target concentration in the solution. By applying this method to a series of different target concentrations, we generate a calibration curve for measuring unknown saturated target concentration. With the target concentration successfully determined, we can easily apply this methodology to determine free and bound species in a wide dynamic range of interacting systems.

With ns-ALEX, we obtain more complete characterization of macromolecular interactions such as fluorophore stoichiometry, internal distance distributions of specific species, fast conformational dynamics of macromolecules, rotational diffusion-related effects, in

addition to determining affinities of interacting species with a wider dynamic range. Also, the addition of FCS allows quantification of labeled molecules on a higher-than-single-molecule concentration level, thus enabling us to measure a broad range of concentrations with the same data acquisition method.

4. Detection of protein-protein interactions in MR-1

With our optical methods in place, the major obstacle to detection of interactions among MR-1 proteins has been producing *functional* MR-1 proteins. Through our collaboration with PNNL, we have received over 20 plasmid clones encoding hexahistidine-fusion (His₆-fusion) of MR-1 proteins. Overexpression of the proteins was achieved in a variety of vectors, but solubility of the proteins is limited in these *E. coli* vectors. We have moved to cytosolic proteins to reduce insolubility and to avoid complex refolding procedures, and as shown in section one, we have successfully expressed and purified both NtrC and σ^{54} . Due to the activator requirement, transcriptional activity of refolded σ^{54} has yet to be determined; however, the NtrC protein was purified from the soluble fraction and can be tested for transcriptional activity using an abortive initiation assay[22-24].

A promoter, glnAp2, which contains two strong NtrC consensus binding sequences was employed for this assay. In this assay a fluorescently labeled nucleotide UTP- γ -AmNS (uridine 5'-triphosphate, P³-(5-sulfo-1-naphthylamide) Molecular Probes), which is quenched as a free nucleotide, is incorporated into the abortive RNA product upon successful transcription. The incorporation of the nucleotide release the fluorophore conjugated to the γ -phosphate, and through the cyclic production of abortive products the fluorescence increases over time. As seen in figure 10B, abortive cycling by σ^{70} shows a dramatic increase in fluorescence over time; however, for MR-1 NtrC (Fig. 10C), there is no increase in fluorescence above the background signal. This negative transcription result may be attributed to the use of *E. coli* RNAP and σ^{54} . Until the full MR-1 RNAP complex can be reconstituted or functional MR-1 σ^{54} verified, we have not produced a functional MR-1 protein in this transcription cascade.

Figure 10. Fluorescent abortive initiation assay (FDAI) **A.** FDAI of *E. coli* holoenzyme ($E \sigma^{70}$) on *E. coli* promoter lacUV5. **B.** FDAI of MR-1 NtrC with *E. coli* RNAP core and σ^{54} on glnAp2 promoter.

In parallel with our work to reconstitute the two-component signaling cascade of NtrC and σ^{54} , we also pursued the reconstitution of an alternative sigma factor in the σ^{70} family, σ^{24} . This sigma factor requires no activating factor and control transcription under extra-cytoplasmic stress or heat shock. As shown in section one, we have successfully overexpressed and purified this protein. To verify activity of this protein, we sought to identify promoters containing the σ^{24} consensus sequences. Using homology comparison with known *E. coli* and *Salmonella typhimurium* σ^{24} promoters[25, 26], we identified two possible promoters for σ^{24} experiments. The first is upstream of the σ^{24} gene itself, rpoE, and the second is upstream of another heat shock sigma factor, rpoH. We cloned these promoter gaps from the MR-1 genome and conducted gel shift assays to verify σ^{24} interaction with *E. coli* RNAP and with the MR-1 promoters (Fig 11A&B). Both promoters indicated positive interactions with the RNAP- σ^{24} ($E\sigma^{24}$) complex. To verify these complexes were not non-specific interactions with other region of the DNA, a heparin-sepharose challenge was used to remove non-specifically bound RNAP complexes, and figure 11B shows that there are specific complexes, which survive heparin challenge. With these positive indications of a functional protein, we are currently in the process of verifying transcriptional activity, and once transcriptional activity has been verified, we will begin interactions studies with the labeled single cysteine mutant of σ^{24} described in section one, and labeled DNA or labeled *E. coli* RNAP.

Conclusions

Over the period of this grant, we have made great advances in the development of optical methods to analyze protein-protein interactions and have overcome many issues involved in the expression and purification of MR-1 proteins. The generous funding provided by this project has lead to the development of a methodology that allows robust analysis of structure and dynamics for a wide range of protein-DNA and protein-protein interactions. With these tools in hand and the production of *functional* MR-1 proteins imminent, we are nearing our objective of unraveling the intricate network of interactions in *Shewanella oneidensis* MR-1.

Publications

Patents

1. Laurence, T.A. and S. Weiss, inventors; UCLA assignee. "Monitoring molecular interactions using photon arrival-time interval distribution analysis." PCT/US03/23252.
2. Kapanidis, A.N., T. Laurence, N.K. Lee and S. Weiss; UCLA assignee. "Alternate laser excitation fluorescence resonance energy transfer (ALEX-FRET): a new method for analysis of macromolecular structure and interactions." US provisional 60/480,346.

Papers

a). Published

1. Kapanidis, A.N., et al., *Fluorescence-aided molecule sorting: analysis of structure and interactions by alternating-laser excitation of single molecules*. Proc Natl Acad Sci U S A, 2004. **101**(24): p. 8936-41.
2. Laurence, T.A., et al., *Photon arrival-time interval distribution (PAID): A novel tool for analyzing molecular interactions*. Journal of Physical Chemistry B, 2004. **108**(9): p. 3051-3067.

b). In press

3. Lee, N. K., A. N. Kapanidis, X. Michalet, E. Margeat, and S. Weiss. *Towards structure from single molecules: Accurate Förster resonance energy transfer measurements using alternating laser excitation*. Biophys J, accepted
4. Kapanidis AN, T. Laurence, N.K. Lee, E. Margeat, X. Kong, and S. Weiss. *Alternating-laser excitation of single molecules*. Acc Chem Res, accepted.

c). In preparation

5. Kapanidis, A.N., E. Margeat, T. A. Laurence, N. K. Lee, J. Mukhopadhyay, E. Kortkhonjia, R. H. Ebright, and S. Weiss. *Single-Molecule Analysis of a σ^{70} release upon promoter escape*.
6. Laurence, T.A., X. Kong, M. Jaeger and S. Weiss. *Structural heterogeneities and conformational fluctuations of biopolymers probed by single molecule nanosecond alternating laser excitation (nsALEX)*

Figure 11. Reconstitution of holoenzyme complex. A. Increasing amounts of 4:1 σ^{24} and RNAP core enzyme ($E\sigma^{24}$) are titrated against rpoE (right) and rpoH (left) operon DNA without heparin challenge. Complexes are indicated by arrow. C. Heparin-sepharose (HS) challenging of complexes. Heparin-sepharose solution is added and removed to reduce non-specific complexes on both rpoE (right) and rpoH (left) DNA. Specific holoenzyme complexes are indicated by arrow.

7. Lee, N.K., H.R. Koh, A.N. Kapanidis, S.O. Ho, N.R. Gassman, S. Weiss, and S.K. Kim. *3-color alternating-laser excitation single-molecule spectroscopy*.
8. Margeat E., A.N. Kapanidis, P. Tinnefeld, Y. Wang, J. Mukhopadhyay, R.H. Ebright, and S. Weiss. *Multi-Path Total-Internal-Reflection Microscopy (MP-TIR) for single-molecule analysis of transcription complexes*.

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