CHAPTER FIVE

Advancing the study of protein-G4 interactions in DNA repair: Insights from biolayer interferometry

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Contents

Abstract

Biolayer interferometry (BLI) is a powerful tool that enables direct observations of protein-G4 interactions in real-time. In this article, we discuss the crucial aspects in conducting a BLI experiment by using the TAR DNA-binding protein (TDP43) and a G4 DNA formed by $(GGGGCC)_4$ as a sample application. We also describe the necessary precautions in designing the DNA substrate and evaluating the signal contributions arising from nonspecific binding interactions. A comprehensive guide is included that details the necessary materials and reagents, experimental procedures, and data analysis methods for researchers who are interested in using BLI for similar studies. The insights provided in this article will allow researchers to harness the potential of BLI and unravel the complexities of protein-G4 interactions with precision and confidence.

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1. Introduction

Biolayer interferometry (BLI) has emerged as a convenient tool for studying molecular interactions in real-time. This approach is akin to surface plasmon resonance (SPR), quartz crystal microbalance (QCM), and other related biosensor techniques in that a molecule of interest is placed on a functionalized surface, and then a binding partner is introduced in solution. The association and dissociation reactions are monitored in different ways. In SPR, changes in the refractive index of the sensor chip surface are used to report binding, while in QCM, interactions of the biomolecules affect the resonance frequency of the quartz crystal (Jost, Munch, & Andersson, 1991; Matsuno, Niikura, & Okahata, 2001). A BLI biosensor functions as a fiber optic cable (Fig. 1) The instrument emits an incident white light that travels through the sensor tip and is reflected back. The interference pattern of the reflected wave is processed and recorded by the instrument. When a partner molecule binds, it effectively increases the optical thickness of the surface or "biolayer" because the light now travels a further distance. This causes a wavelength shift in the interference pattern of the white light, and that difference in signal (i.e. $\Delta\lambda$; measured in nm) is used to observe either complex formation or disassembly (Ciesielski, Hytonen, & Kaguni, 2016). In this article, we will highlight the advantages and limitations of BLI, and discuss the important considerations when designing an experiment. We will demonstrate a sample application of BLI to study the interactions between the TAR DNA-binding protein 43 (TDP43) and a G-quadruplex formed by a hexanucleotide repeat sequence $(GGGGCC)_4$.

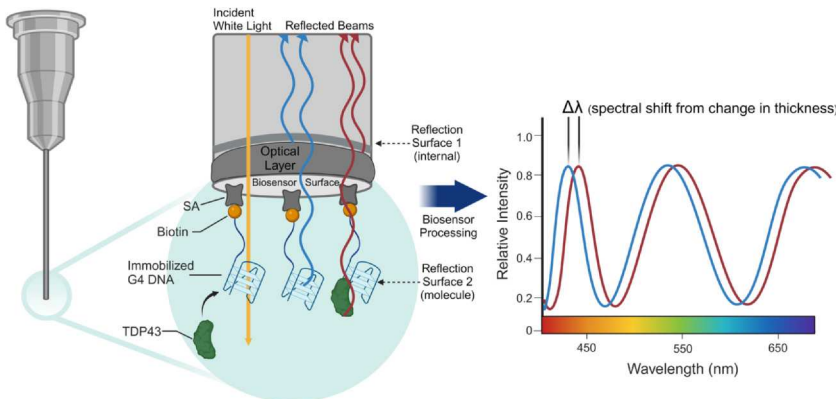

Fig. 1 BLI overview.

G-quadruplexes (G4s or GQs) are stable secondary structures that are formed by guanine-rich DNA or RNA (Bochman, Paeschke, & Zakian, 2012). G4s are not only ubiquitous in cells, but also play critical roles in regulating gene expression, telomere maintenance, and other cellular processes (Gray, Vallur, Eddy, & Maizels, 2014; Schulz & Zakian, 1994; Siddiqui-Jain, Grand, Bearss, & Hurley, 2002). Because G4s fold spontaneously when single-stranded DNA (ssDNA) is exposed, they can interfere with DNA replication, recombination, and repair if they accumulate in cells. There has been ongoing research interest in understanding how repair enzymes as well as other proteins target and/or unfold G4s. For example, the $(GGGGCC)_4$ hexanucleotide repeat expansion (HRE) is found in a noncoding region of C9ORF2 (chromosome 9, open reading frame 72) and it is implicated as a major genetic cause of frontotemporal dementia (FTD) and amyotrophic lateral sclerosis (Ash et al., 2013). This HRE sequence adopts a G4 in the RNA transcript that can become a substrate for repeat-associated non-ATG (RAN) translation (Cleary, Pattamatta, & Ranum, 2018). TDP43 has been proposed to play a key role in regulating this process and in trafficking the G4-containing mRNA (Ishiguro & Ishihama, 2023; Ishiguro, Kimura, Watanabe, Watanabe, & Ishihama, 2016). However, the precise mechanisms by which TDP43 functions are not well-understood. The protein possesses two RNA-recognition motifs (RRM1 and RRM2), which have distinct nucleic acid recognition properties (Furukawa et al., 2016). RRM1 facilitates interactions with ssRNA or ssDNA, while RRM2 provides the protein with the specificity needed to target UG or TG-rich sequences. These functions are consistent with TDP43 acting on the UG-repeats at the splice sites of pre-mRNA. Because TDP43 binds not only to single-stranded nucleic acids, but also to G4 RNA/DNA, BLI is a useful tool with which to determine the relative substrate binding specificities of its targets. Here, we describe a BLI assay for monitoring the interactions between TDP43 and a G4 DNA formed by $(GGGGCC)_4$.

2. Major considerations

BLI requires one of the binding partners to be immobilized on a biosensor surface. The first step in designing an experiment is to determine which species to place on the sensor. Data will have a better signal-to-noise ratio when the larger molecule is used as the analyte (i.e. free in solution),

as there is a greater signal change in "optical thickness" when it binds. The capability of the equipment available also presents an experimental constraint. Currently, Sartorius is the sole source for BLI instrumentation and consumable biosensors. The entry-level Octet N1 has a recommended detection limit of ~10 kDa; therefore, the analyte should have a molar mass of at least that size for reliable measurements. This is still feasible for studying the binding of proteins and short DNA/RNA oligos, but excludes the analysis of nucleotide cofactors/analogs, small molecule inhibitors, and other pharmacological ligands. For such applications, the Octet R-Series instruments are capable of measuring size changes of just ~150 Da. A key advantage of BLI over other related techniques is its simple dip-and-read system, eliminating the need of microfluidics for sample delivery. The biosensors are moved across different tubes or microplate wells for each measurement. This open format is more user-friendly, the equipment is easier to maintain, and it enables the analysis of crude samples such as blood, serum, and cell extracts.

Another major consideration is to determine *how* the biomolecule will be attached to the surface. BLI sensors are commercially available for a wide range of applications. Streptavidin-coated sensors are used to capture biotinylated molecules, Ni-NTA sensors can immobilize His-tagged proteins, and anti-glutathione-*S*-transferase (GST) sensors can bind to GST-tagged proteins, etc. When characterizing a protein-G4 DNA interaction, streptavidin sensors are commonly used because the DNA substrate can be synthesized with a biotin modification. A flanking linker region is incorporated into the oligo to provide additional flexibility; otherwise, its surface orientation may occlude the protein partner from binding.

Nonspecific binding of the analyte can lead to incorrect interpretations of the BLI data. There are two main sources of nonspecific interactions. The freely diffusing protein may adsorb onto the biosensor surface directly to produce a false signal or there may be other binding sites on the immobilized G4 substrate. To test for nonspecific adsorption, BLI traces are collected as a function of protein concentration on the streptavidin sensor in the absence of the biotinylated DNA. If a protein-dependent binding signal is observed, then 0.1–1% bovine serum albumin is included in the assay buffers to mitigate the effect, and any nonspecific binding signal contributions are subtracted from the G4-binding time-courses. Additionally, the DNA substrate should be designed to limit the number of off-target interaction sites. For instance, if a protein has affinity for both ssDNA and G4, then ssDNA would not be an appropriate handle to use

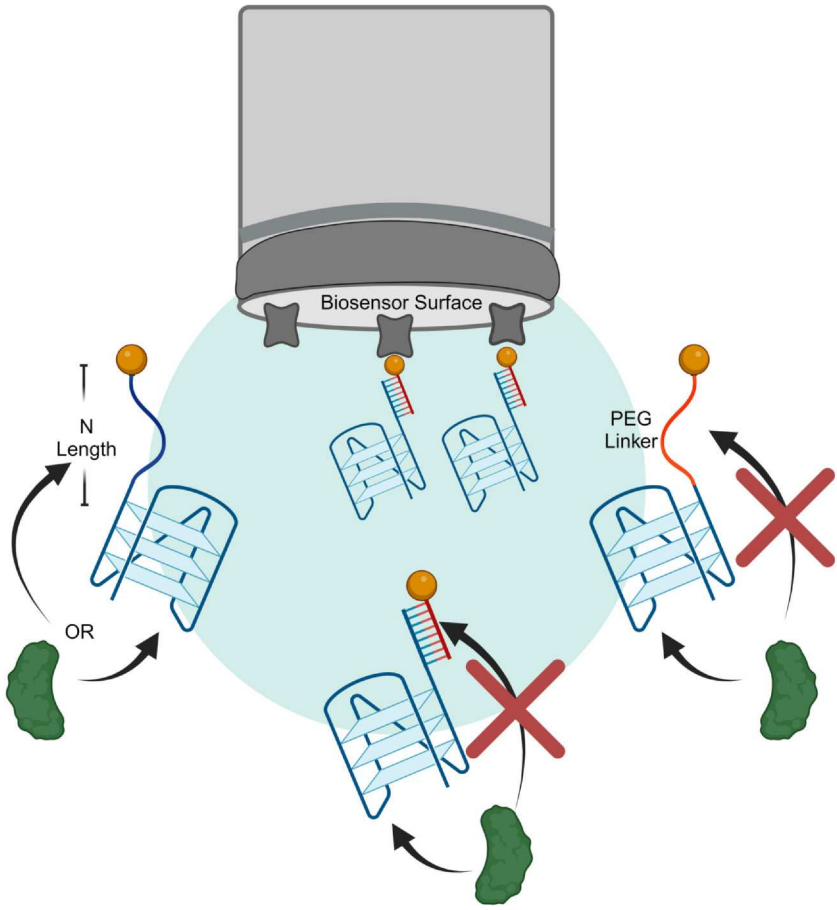

Fig. 2 DNA substrate design.

because it will introduce an additional binding site (Fig. 2). The length of the DNA handle (N) can be reduced to prevent the protein from binding, a complementary duplex can be introduced if the protein does not interact with dsDNA, or the ssDNA handle can be replaced entirely with a polyethylene glycol (PEG). The PEG linker is designed to have the same contour length as ssDNA, but it lacks the electrostatic contacts of the phosphodiester backbone and therefore reduces the interactions with nucleic acid-binding proteins (Wong, Rice, Baker, Ju, & Lohman, 2006; Wu & Lohman, 2008). Control BLI experiments are performed with the biotinylated linker (without the G4) to confirm that the protein does not bind directly to the handle.

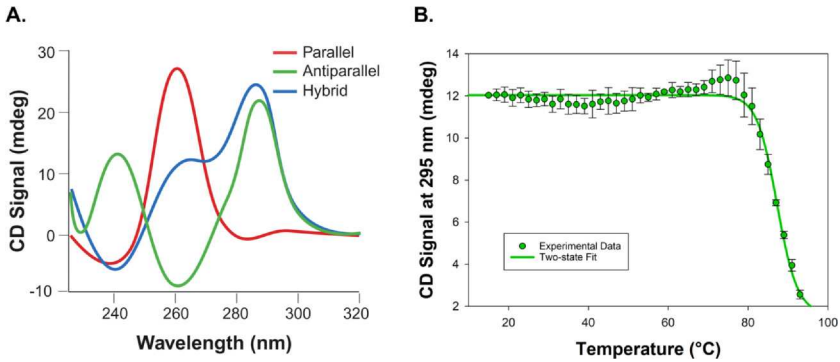

Fig. 3 Assessing G4 conformation and thermal stability by circular dichroism spectroscopy. (A) CD characteristics of parallel, antiparallel, and hydrid G4 DNA. (B) Thermal denaturation of a hydrid G4.

Lastly, because this article focuses on studying the interactions between TDP43 and G4 DNA by BLI, one must independently verify that the G4 is folded under the assay solution conditions. Circular dichroism (CD) spectroscopy is an effective method for monitoring the folding of G4s. G4s can adopt parallel, antiparallel, or hybrid spatial configurations that have distinct CD characteristics (Fig. 3A) (Del Villar-Guerra, Gray, & Chaires, 2017; Del Villar-Guerra, Trent, & Chaires, 2018); hence, CD spectra validates successful formation of the G4 prior to the BLI experiments. By collecting the data as a function of temperature and monitoring the loss of its signature CD signal as the G4 unfolds, the thermal stability of the G4 (i.e. T_m) can be determined (Fig. 3B).

3. Material and equipment

3.1 General equipment

- Sartorius Octet N1 (formerly "BLItz") or Octet R8 BLI instrument
- BLItz Pro instrument software (for the Octet N1) or CFR software (for the Octet R8)
- Sartorius high precision streptavidin (SAX) biosensors*
- Eppendorf Safe-Lock 0.5 mL amber microcentrifuge tubes (if using the Octet N1)
- Greiner black 96-well microplates (if using the Octet R8)
- GraphPad Prism or similar software for data analysis

*Sartorius offers streptavidin (SA) as well as high precision streptavidin (SAX and SAX 2.0) biosensors. SAX sensors are tested for lot-to-lot variations. BLI data collected and replicated from sensors that are manufactured within the same lot number are expected to have within 4% agreement, while data compared from across different lots can deviate up to 20%.

3.2 Protein and DNA

- G4 reaction buffer (20 mM HEPES (4-(2-hydroxyethyl)− 1-piperazineethanesulfonic acid) pH 7.5, 150 mM KCl, 5 mM TCEP-HCl (tris (2-carboxyethyl)phosphine hydrochloride), 20% glycerol)
- DNA oligonucleotides (synthesized by integrated DNA technologies)
  - Top strand: 5′-bio poly T16-3′ (5′-biotin-TTTTTTTTTTTTTTTT-3′)
  - Bottom strand: 5′-C9FTD-poly A16-3′ (5′-GGGGCCGGGGCCG GGGCCGGGGCC-AAAAAAAAAAAAAAAA-3′)
- Purified TDP43 protein

The DNA substrate is annealed in G4 reaction buffer by mixing the top and bottom strands, with the non-biotinylated DNA in slight molar excess (1.05 times). The sample is placed in a 95 °C heating block for 10 min and then cooled slowly to 25 °C over three hours. Successful annealing of the DNA is confirmed by 10% PAGE and Stains-All (Sigma Aldrich), and formation of the G4 is validated by CD spectroscopy.

4. BLI binding assay

4.1 Sample preparation (for the Octet N1)

1. Label the amber tubes: B, D, or [P] (B—Buffer; D—DNA; [P]—TDP43 protein concentration). The Octet N1 requires ~300 μL of sample material in a 0.5 mL tube to measure the signal reliably. Each BLI run requires three B tubes, one D tube, and a P tube. The number of runs per trial is dependent on the protein concentration range needed to obtain a complete binding isotherm. This will be discussed below in the Data Analysis section. Note that the final run will be a buffer only reference (0 μM protein).
2. Transfer 300 μL of G4 reaction buffer to each B tube; these will be used for collecting baselines and for washing the biosensors. Transfer 300 μL

of buffer to all of the protein tubes except for the top end concentration (12 μM), which will be added directly from the stock. These tubes will be used to set up a dilution series.

3. Dilute the annealed DNA stock to 150 nM. Pipette 300 μL of the DNA into each of the D tubes. There should be a DNA tube for every BLI run (i.e. each analyte concentration plus the buffer reference). Aliquoting the DNA this way improves data reproducibility because the contents of each D tube will be identical.
4. Thaw the TDP43 protein stock on ice for 10–20 min.
5. Prepare 650 μL of 12 μM TDP43.
6. Transfer 300 μL of the stock protein into an empty P tube labeled "12 μM." This will be the top end or the highest analyte concentration in the experiment.
7. Transfer 300 μL of the remaining 12 μM TDP43 to a P tube labeled "6 μM" (which already contains 300 μL of buffer) and pipette mix gently, then remove 300 μL of that solution for the next tube in the dilution series. Continue with this process until the final desired concentration of protein is reached. Do not add any protein to the final reference tube (buffer only) (Fig. 4).

4.2 Running the BLI experiment

1. Transfer 300 μL of G4 reaction buffer into the wells of a biosensor tray. For each run, hydrate the same number of streptavidin-coated biosensors (SAX) in the buffer for a minimum of 10 min.
2. The Octet R8 has a temperature-controlled climate chamber but the Octet N1 does not. When performing experiments with the N1, sample

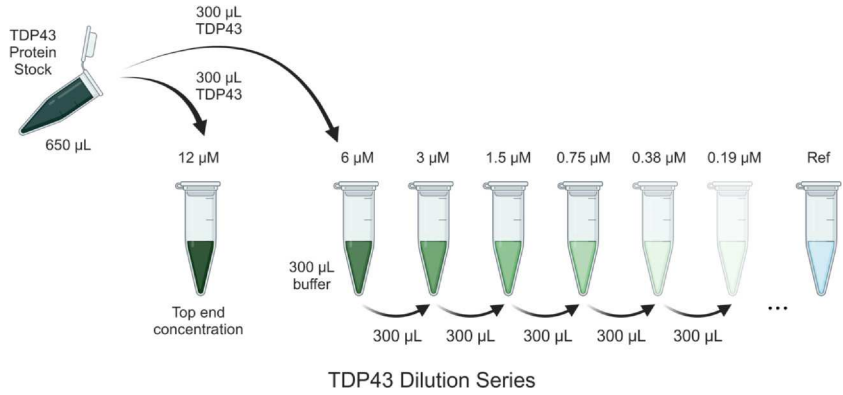

Fig. 4 Preparation of a protein dilution series.

tubes are kept at 25 °C and 250 rpm in an Eppendorf ThermoMixer until they are placed in the BLI instrument for immediate measurements.

3. In the Blitz Pro program, select Advanced Kinetics. Switch all steps to Tubes. Adjust the association and dissociation steps to 180 s each.
   a. The association and dissociation times are unique to each protein. After an initial experiment, these parameters are adjusted to allow for sufficient time for the protein-DNA binding signal to reach a plateau and for protein dissociation to complete.
   b. Confirm that all steps are set for Tubes if using the amber tubes. Alternatively, the Drops option may be selected in applications where only ~4 μL of analyte sample is available. The protein aliquot would then be loaded onto a drop holder accessory for BLI measurements.
4. Click Run. Load a hydrated SAX biosensor and a buffer tube to begin each run. The N1 instrument will prompt the user to switch tubes at the next step. BLI has a dip and read format and the biosensor is directly dipped into the sample tubes for real-time analysis.
5. An overview of a typical BLI experiment is shown in Fig. 5A. First, the SAX sensor is placed in the instrument and the interference pattern of the incident light is recorded in buffer alone (Phase 1). Next, the biotinylated DNA substrate is loaded onto the sensor through its

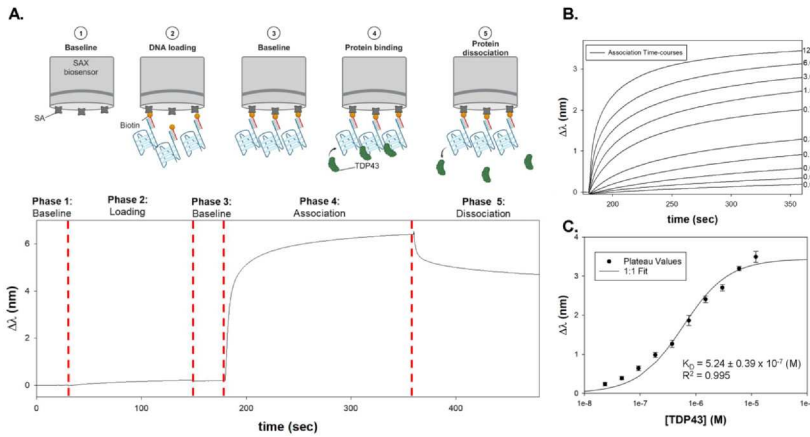

Fig. 5 BLI data collection and analysis. (A) Cartoon representation of the individual steps of a typical BLI experiment. (B) Truncated BLI time-courses of the association phases as a function of analyte concentration. (C) Non-linear least squares analysis of the steady-state BLI signals to a 1:1 binding model.

interactions with the streptavidin (Phase 2). An increase in the optical thickness at the sensor tip upon DNA binding produces a wavelength shift to the interference pattern ($\Delta\lambda$ measured in nanometers; y-axis). The biosensor is washed with buffer to remove any free DNA that is not bound (Phase 3). A stable second baseline indicates that the G4 DNA remains bound to the sensor. Freely-diffusing TDP43 is then introduced (Phase 4). Formation of protein-DNA complexes further enhances the optical interference. After a plateau is reached, the sensor is returned into buffer, and a decrease in binding signal is observed as TDP43 dissociates from the G4 (Phase 5). This process is repeated with a fresh biosensor for each TDP43 analyte concentration and for the final reference in buffer alone.

4.3 Data processing

1. Once data collection is complete, scroll down to the Run List window and check the Ref. box to indicate which dataset corresponds to the reference run. The software will automatically subtract this signal from the other curves as indicated by the time-courses shown in Fig. 5B. Scroll down to the graph in the Analysis Data window. Click the Analyze button, then right click the graph. Click Export Data and export the data as a text file.
2. Open the .txt file in Excel. Each experiment produces text data listed in ~15,000 rows. Using the Find command (CTRL+F), jump to each run (i.e. protein analyte concentration), copy those values into a separate Excel spreadsheet, and organize the data by TDP43 concentration.

4.4 Data analysis

1. BLI data can be analyzed in one of two ways. First, the BLI time-courses are globally analyzed in GraphPad Prism software using the following equations which describe a simple 1:1 binding model (Bjorquist & Bostrom, 1997).

$$R = (R_0/k_{obs})(1 - e^{-k_{obs}(t-t_0)}) \quad (1)$$

where

$$k_{obs} = k_{on}[TDP43] + k_{off}$$

$$R = R_0 e^{-k_{off}(t-t_0)} \quad (2)$$

The association phases are fit to Eq. (1), in which the binding response signal (R) is described by an exponential increase function with time constant k_{obs}. The dependence of k_{obs} on TDP43 concentration is used to determine the association rate constant k_{on}. The dissociation phases are analyzed by an exponential decay function, Eq. (2). Since the breakdown of the TDP43-G4 complex is a unimolecular process, the dissociation rate constant k_{off} is independent of protein concentration. The ratio of the two rate constants is used to calculate the affinity for the TDP43-G4 interaction ($K_D = k_{off}/k_{on}$).

2. Alternatively, the steady-state plateau values of the association phases are plotted against total analyte concentration to generate a binding isotherm (Fig. 5C). The plateau region of each time-course is identified within the dataset, and the last 100 data points are averaged to determine the steady-state binding signal. This data is fit to Eq. (3) for a simple one-site binding model, where the y-values are the plateau signals, A is an amplitude term, D is the G4 DNA concentration (150 nM), P is the TDP protein concentration, and K_D is the equilibrium dissociation constant (Lowran, Campbell, Popp, & Wu, 2019).

$$y = A * \frac{(K_D + D + P) - \sqrt{(K_D + D + P)^2 - 4DP)}}{2D} \tag{3}$$

3. To assess data reproducibility, BLI experiments are repeated in triplicates. The average K_D and standard error values within a 95% confidence interval are reported.

5. Conclusions

In summary, BLI provides real-time analysis of protein-G4 association and dissociation reactions. In comparison to SPR and QCM, BLI excels in its simplicity and versatility. Proper precautions should be taken in designing of the DNA substrate and assessing the signal contributions from nonspecific binding. If possible, BLI results should be validated by an independent method that does not require surface immobilization (e.g. isothermal titration calorimetry, fluorescence anisotropy, etc.) before conducting the assays in high throughput. CD is often used in conjunction with binding assays to determine the G4 conformations under

the assay conditions, but it should be noted that G4-folding (and unfolding) cannot be monitored by BLI directly. However, with its dip-and-read capability and diverse sensor options, BLI continues to be a valuable tool for bioanalytical research by offering detailed insights into macromolecular interactions.

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