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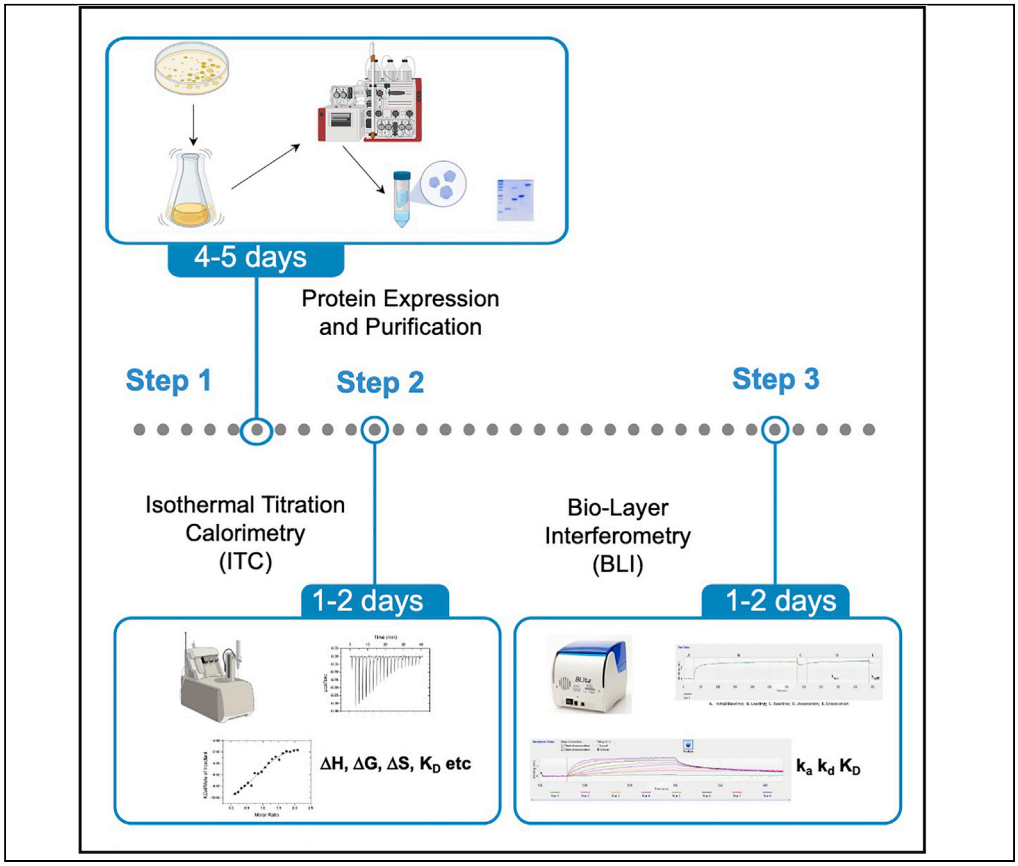
Protocol

Label-free protocol to quantify protein affinity using isothermal titration calorimetry and bio-layer interferometry of a human eIF5-mimic protein



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Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Label-free protocols to quantify protein affinity

Isothermal titration calorimetry or ITC titrates heat released by ligand injection

Bio-layer interferometry or BLI titrates sensogram responses by ligand binding

Protocols for protein purification by nickel-affinity chromatography are included

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Label-free protocol to quantify protein affinity using isothermal titration calorimetry and bio-layer interferometry of a human eIF5-mimic protein

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SUMMARY

eIF5-mimic protein (5MP) controls translation through its interaction with eukaryotic translation initiation factor (eIF) 2 and eIF3 and alters non-AUG translation rates for oncogenes in cancer and repeat expansions in neurodegenerative disease. To precisely evaluate the effect of 5MP mutations on binding affinity against eIFs, here we describe two label-free protocols of affinity measurement for 5MP binding to eIF2 or eIF3 protein segments, termed isothermal titration calorimetry (ITC) and bio-layer interferometry (BLI), starting with how to purify proteins used.

For complete details on the use and execution of this protocol, please refer to Singh et al. (2021).

BEFORE YOU BEGIN

Translation initiation in eukaryotes is a complex process involving 5′-terminally capped mRNA, ribosome, Met-tRNA$_i^{Met}$ and eukaryotic translation initiation factors (eIF) 1, 1A, 2, 3, 4F, 5 and 5B (Asano, 2014; Asano et al., 2001; Hinnebusch et al., 2007). As a molecular mimic of the C-terminal part of the eIF5, eIF5-mimic protein regulates translation initiation (Singh et al., 2011). Of note, recent studies highlight its ability to modulate translation initiation frequencies from near-cognate (non-AUG) start codons for oncogenes in cancer (Sato et al., 2019; Tang et al., 2017) and for repeat-expansion in neurodegenerative disease (Singh et al., 2021). Here we describe two methods of label-free affinity measurement for 5MP binding to its binding partners in the β subunit of eIF2 and the c subunit of eIF3, using isothermal titration calorimetry (ITC) and bio-layer interferometry (BLI). To dissect the interaction of human 5MP1 (h5MP1), one of the two copies of 5MP found in Homo sapiens, we used two mutations altering acidic and basic surfaces of its C-terminal domain termed 7A (Singh et al., 2011) and BN1 (Singh et al., 2021). We also used the minimal segments of eIF2β (Luna et al., 2012) and eIF3c (Singh et al., 2021) termed heIF2β$_{53\text{-}136}$ and heIF3c$_{20\text{-}102}$, respectively, determined to be responsible for their binding to h5MP1.

In ITC, highly purified molecules (proteins, nucleic acids or ligands) are mixed together, and the interaction between the molecules in the system is determined by the amount of heat released

(Pierce et al., 1999). The isothermal titration calorimeter, or the ITC machine, has two cells, one for reference and the other with samples. A fixed amount of a molecule is loaded into the sample cell, to which increasing amounts of the other molecule is added for a titration experiment. The output profile, a thermogram, is expected to display a sigmoidal curve, which allows the calculation of the dissociation constant (K_D) for the binding affinity and stoichiometry (N) between the two molecules.

Workflow of the ITC assay:

1. Dilute highly purified proteins to a fixed concentration (typically between 1 μM to 1 mM, depending on K_D of their binding).
2. Wash Cell & Syringe of the ITC instrument (MicroCal ITC_{200}) thoroughly with deionized water followed by size-exclusion buffer.
3. Fill sample cell with 200 μL of a protein and pipette/injector with 40 μL its binding partner.
4. Titrate samples at an equilibrium temperature of 25°C under a program setting.
5. Process data sets and plotted the results using Origin software.

The BLI (e.g., the BLItz system from ForteBio) detects in real-time the dynamic changes in reflectance interference wave pattern due to the change in protein mass on the biosensor tip (Rich and Myszka, 2007). Among the many methods of immobilizing a binding molecule to the biosensor tip, the anti-GST biosensor was chosen due to the convenience of one-step immunoaffinity-purification of glutathione-S-transferase (GST)-fusion proteins.

Workflow of the BLI assay (onscreen instructions were prompted by the software):

6. Take Initial Baseline using 200 μL of appropriate binding buffer for 30 s.
7. Load the appropriate GST fusion protein on the Anti-GST Biosensor in 4 μL for 120 s as you observe loading curve in real time.
8. Take another Baseline measurement using 200 μL of BLI binding buffer for 30 s.
9. Perform association with the analytes (binding partner) using 4 μL of known concentration of the analytes for 120 s; it provides the binding/association curve.
10. Conduct dissociation step in 250 μL of BLI binding buffer for 120 s so that the dissociation curve can be generated.

Successful experiments critically depend on the quality (in most cases, purity) of the proteins used. Below we describe the preparation of the materials established in our labs in detail, which were used for these label-free assays reported in (Singh et al., 2021).

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
E. coli BL21 (DE3), chemically competent cells	EMD Biosciences	69451
Chemicals, peptides, and recombinant proteins		
Agar	Fisher Scientific	BP1423-500
Ampicillin	GoldBio	A-301-100
Ammonium persulfate	Sigma-Aldrich	A-6761
β-Mercaptoethanol	Sigma-Aldrich	M6250-250
Bromophenol Blue	Sigma-Aldrich	B8026-5GM
BSA	Pierce Thermo Scientific	23209; 2 mg/mL
DTT	Sigma-Aldrich	D9779
EDTA	Fisher Scientific	S311-500

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Glycine	Sigma-Aldrich	G7126-5KG
Glutathione Sepharose 4B	Sigma-Aldrich	Cytiva 17-0756-01
Ethanol	Decon Labs, Inc.	2701 1 GAL.
Glycerol	Fisher Scientific	BP229-1
HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)	GoldBio	H-401-500
Hydrochloric Acid	Fisher Scientific	A-144S-500
Imidazole	Fisher Scientific	O3196-500
Isopropyl β-d-1-thiogalactopyranoside (IPTG)	GoldBio	12481C-50
Kanamycin monosulfate	GoldBio	K-120-25
Luria Bertini (LB) media Miller	Fisher Scientific	BP-1426-2
Sodium chloride (NaCl)	Research Products International	523030-12000
Sodium dodecyl sulfate (SDS)	Sigma-Aldrich	L3771-500
Sodium hydroxide (NaOH)	Fisher Scientific	SS255-1
TCEP	GoldBio	TCEP25
TEMED	Bio-Rad	161-0800
TGX-stain free fast cast Acrylamide 12% kit	Bio-Rad	1610185
Tris Base	Research Products International	T60040-1000
Tween 20	Fisher Scientific	BP337-500
Recombinant DNA		
pET28 a/b/c (+)	EMD Biosciences	69864
pET30 GBFusion1	Gerhard Wagner	N/A
pET–h5MP1	(Hiraishi et al., 2014)	Asano lab p1479
pET–h5MP1-7A	(Singh et al., 2021)	Asano lab p1957
pET–h5MP1-BN1	(Singh et al., 2021)	Asano lab p1958
pGEX-h5MP1	(Singh et al., 2011)	Asano lab p1535
pGEX-h5MP1-7A	(Singh et al., 2011)	Asano lab p1116
pGEX-h5MP1-BN1	(Singh et al., 2021)	Asano lab p1818
pGB-heIF2β-K2K3	(Luna et al., 2012)	Asano lab p1336
pGB-heIF3c12-His	(Singh et al., 2021)	Asano lab p1837
Software and algorithms		
Origin software	MicroCal	N/A
BLItz Pro software	ForteBio	N/A
Other		
Amicon Ultra Centrifugation Unit	Millipore	UFC901024
Anti-GST biosensors	ForteBio	18-5096
Autoclave	STERIS	Amsco Lab 250
Baffled Shaker Flasks, 2 Liters	KIMAX Kimble	25630
Bottle Top Filter	CELLTREAT Scientific Products	229716
BLItz	ForteBio	45-5000
Centrifuge	Thermo Scientific	Sorval ST 16R
Centrifuge Bottles (1 Liter)	Beckman Coulter	366751
Centrifuge Tubes (50 mL)	Genesee Scientific	28-108
Deionized Water Unit	Thermo Scientific	7119
Disc filter membrane	Millipore	GPWP04700
Dialysis membrane tube	Thermo Scientific	68100
Dialysis membrane clips	Thermo Scientific	68011
FPLC	Cytiva (Previously GE)	Akta Pure
Fraction collection tubers	Fisher Scientific	14-96-27
Glassware/plasticware	N/A	N/A
Hamilton Syringe	Hamilton	Syn50018P
High speed centrifuge	Beckman Coulter	Avanti J-26XP
High speed centrifuge rotor	Beckman Coulter	JLA 8.1
High speed centrifuge rotor	Beckman Coulter	JA 30.5
HiLoad 16/600 Superdex 75 pg Column	Cytiva (Previously GE)	289893-33

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Incubator	Labline Instruments Inc	Imperial III
Incubator Shaker	New Brunswick Scientific	Excella E24
Incubator Shaker	New Brunswick Scientific	I2500 Series
Isothermal Titration Calorimetry Instrument	Malvern	MicroCal iTC200
Nano Drop UV Spectrophotometer	Thermo Scientific	NanoDrop One
Loop/Spreader		N/A
Oak Ridge PSF tubes	Nalgene	3115-0050
Petri dishes		N/A
Protein Electrophoresis Equipment	Bio-Rad	PowerPac Basic
Protein Electrophoresis Running Reservoir	Bio-Rad	MiniProtean Tetra Cell
ProteIndex Hibond Ni-NTA Agarose 6 Fast Flow Resin	Marvelgent Biosciences	11-025-050
Sonicator	Misonix	S-4000
Sonicator Stainless Steel container	N/A	N/A
Spectrophotometer	Amersham Biosciences	Ultraspec 10
Syringe Filter	Sarstedt	83.1826.001
−80°C Ultradeep Freezer	New Brunswick Scientific	U535 Innova

MATERIALS AND EQUIPMENT

LB Media:		
Reagent	Final concentration	Amount
LB Media Miller	N/A	25 g
diH_2O	N/A	1 liter
Total	N/A	1 liter

- Weigh required amount of LB (25 grams X n, where n liter of media) and dissolve in deionized water (n liters).
- Pour 1 liter using a measuring cylinder in 2 L Erlenmeyer flask and autoclave (using the 20 min sterilization cycle at 121°C and 20 psi).
- Store at room temperature if used within 12 h; otherwise, store at 4°C for maximum duration of 1 year.

LB Agar Plates		
Reagent	Final concentration	Amount
LB Media Miller	N/A	25 g
Agar	1.5% w/v	7.5 g
diH_2O	N/A	1 liter
Total	N/A	500 mL
Antibiotic Stock: Ampicillin 200 mg/mL; Kanamycin 25 mg/mL.		

Petri plates/dishes: 20–25.

- Weigh required amount of LB and agar powder. Dissolve in deionized water.
- Pour 1 liter in 1-liter autoclavable bottle and autoclave (using the 20 min sterilization cycle).
- Let the media cool to 50°C, add appropriate antibiotic, mix (Final concentrations: Ampicillin 100 μg/mL; Kanamycin 50 μg/mL).
- Pour 20–25 mL in petri plates.
- Let it set and store at 4°C in dark for maximum duration of 3 months.

Note: Let the media cool down to ~55°C before adding antibiotics to it. LB Agar Ampicillin or Kanamycin plates can be stored at 4°C for the maximum duration of 2–3 weeks.

Note: Prepare LB agar plates (with appropriate antibiotics) & LB media in advance.

10× SDS-PAGE Running Buffer (1 Liter)

Reagent	Final concentration	Amount
Tris base:	0.25 M	30.3 g
Glycine	1.924 M	144.4 g
SDS	1% w/v	10 g
ddH_2O	N/A	To make volume 1 liter
Total	n/a	1 liter

- Fill beaker with 800 mL of dH_2O and place on stir plate with stir bar.
- Weigh Tris base & Glycine and add with stirring, allow to dissolve.
- Weigh SDS and add slowly to mix thoroughly.
- Bring to 1-liter final volume with dH_2O.
- Store in a glass bottle.at room temperature for maximum duration of 1 year.

Note: The pH of buffer should be 8.1–8.3 and there's no need of pH adjustment. 10× running buffer can be stored at room temperature for long time. Dilute to 1× using diH_2O prior to use for running gels.

Note: Mix-in SDS slowly while stirring to avoid frothing.

5× Protein Loading Buffer (Laemmli Buffer) (10 mL)

Reagent	Final concentration	Amount
1 M Tris pH 6.8	0.25 M	2.5 mL
Glycerol	50% w/v	5 mL
SDS	10% w/v	1 g
Bromophenol Blue	0.25% w/v	25 mg
β-mercaptoethanol	25% v/v	2.5 mL
diH_2O	N/A	To make volume 10 mL
Total	N/A	10 mL

- Add Tris and glycerol in a beaker and place on stir plate with stir bar.
- Mix in slowly SDS and let it dissolve.
- Add Bromophenol blue to the solution.
- Aliquot and store at room temperature or 4°C for maximum duration of 1 year.

Note: Add β-mercaptoethanol (final 5%) to aliquot prior to use. Once you add β-mercaptoethanol, store aliquot at −20°C.

8× Binding Buffer (1 Liter)

Reagent	Final concentration	Amount
Tris base:	160 mM	19.38 g
NaCl	500 mM	29.22 g
imidazole	40 mM	2.72 g
ddH_2O	N/A	To make volume 1 liter
Total	N/A	1 liter

- In a beaker add 800 mL dH_2O and place on stir plate with stir bar.
- Weigh all chemicals and add to dH_2O.
- Bring volume to 1 liter, transfer to glass bottle and store at room temperature for maximum duration of 1 year.

Note: Prior to use, dilute to 1× with cold diH_2O, add TCEP (1 mM final concentration) and adjust pH to 8.0. Filter with 0.22 μm filter and store at 4°C.

4× Elution Buffer (1 Liter)		
Reagent	Final concentration	Amount
Tris base:	160 mM	19.38 g
NaCl	500 mM	29.22 g
imidazole	4 M	272.32 g
ddH_2O	N/A	To make volume 1 liter
Total	N/A	1 liter

- In a beaker add 800 mL dH_2O and place on stir plate with stir bar.
- Weigh all chemicals and add to dH_2O.
- Bring volume to 1 liter, transfer to glass bottle and store at room temperature for maximum duration of 1 year.

Note: Dilute to 1× with cold diH_2O, add TCEP (1 mM final concentration) and adjust pH to 8.0. Filter with 0.22 μm filter and store at 4°C.

Size Exclusion Buffer (1 Liter)		
Reagent	Final concentration	Amount
HEPES	10 mM	2.38 g
NaCl	125 mM	7.3 g
TCEP	1 mM	0.28 g
ddH_2O	N/A	To make volume 1 liter
Total	N/A	1 liter

- In a beaker add 800 mL dH_2O and place on stir plate with stir bar.
- Weigh all chemicals and add to cold dH_2O.
- Adjust pH to 7.5 using HCl or NaOH.
- Bring volume to 1 liter, transfer to glass bottle and store at 4°C for maximum duration of 1 year.

Note: All chemical can be prepared in advanced but TCEP should be added before use with adjustment of solution pH.

Thrombin Dialysis Buffer		
Reagent	Final concentration	Amount
Tris base	20 mM	2.42 g
NaCl	150 mM	8.77 g
TCEP	1 mM	0.28 g
ddH_2O	N/A	To make volume 1 liter
Total	N/A	1 liter

- In a beaker add 800 mL dd H_2O and place on stir plate with stir bar.
- Weigh all chemicals and add to cold dd H_2O.
- Adjust pH to 8.0 using HCl.
- Bring volume to 1 liter, transfer to glass bottle and store at 4°C.

Phosphate Buffered Saline (PBS) (1 Liter)		
Reagent	Final concentration	Amount
NaCl	137 mM	8 g
KCl	2.7 mM	0.2 g
Na_2HPO_4	10 mM	1.44 g
KH_2PO_4	1.8 mM	0.24 g
ddH_2O	N/A	To make volume 1 liter
Total	N/A	1 liter

- In a beaker add 800 mL dH_2O and place on stir plate with stir bar.
- Weigh all chemicals and add to cold dH_2O.
- Adjust pH to 7.4 using HCl.
- Bring volume to 1 liter, transfer to glass bottle and store at 4°C. Good for 1 year.

BLI Binding Buffer (PBS-based) (10 mL) - Make this prior to the assay		
Reagent	Final concentration	Amount
1× PBS	1×	10 mL
Tween 20	0.05%	5 µL
BSA (2 mg/mL)	0.0005%	0.25 µL
1 M DTT	0.1 mM	10 µL

STEP-BY-STEP METHOD DETAILS

Timing: 5 days for h5MP1 protein purification

Timing: 1.5–2 h for Day 1

Timing: 16–18 h for Day 2

Timing: 5 h for Day 3

Expression & purification of human 5MP1 (h5MP1) and its binding partners heIF2$\beta_{53\text{-}136}$ and heIF3$c_{20\text{-}102}$.

1. h5MP1 protein purification.

Day 1.

a. Transformation of BL21 (DE3) E. coli cells with pET-h5MP1 (Wild Type/BN1/7A) and heIF2$\beta_{53\text{-}136}$ and heIF3$c_{20\text{-}102}$.
   i. Take a vial of competent cells and thaw on ice.

Note: Make sure to thaw competent cells on ice and handled gently as sudden changes of temperature affects cells viability.

   ii. Add <100 ng of plasmid to the competent cells.

iii. Incubate cells-DNA mixture on ice for 30 min.
iv. Heat shock competent cells at 42°C for 30 s.
v. Add 300 μL of sterile LB broth to the tube and incubate at 37°C in a shaker incubator at 220 rpm for 1 h.
vi. Plate 60 μL of cells onto LB agar plates with 50 μg/mL Kanamycin and place the plates in an incubator overnight (16–18 h) at 37°C.

Day 2.

b. Pre-culture for h5MP1, heIF2$\beta_{53\text{-}136}$ and heIF3c$_{20\text{-}102}$.
i. Inoculate a single transformed colony into 100 mL LB media with Kanamycin (50 μg/mL) and incubated in shaker at 220 rpm at 37°C overnight.

Day 3.

c. Large scale cultures.
i. Inoculate 20 mL of the overnight pre culture into 1 L LB media with Kanamycin (50 μg/mL) and incubate in shaker at 220 rpm at 37°C till OD_{600}=0.6.
ii. Induce cell culture for T7 RNA polymerase expression with 0.5 mM IPTG final concentration and incubate further for 3–4 h.
iii. Harvest cells by centrifugation at 14000 g (Beckmann JLA 8.1 Rotor) for 15 min at 4°C and store pellets in the −80°C freezer.

Note: Maintain sterile condition while working with bacterial culture to avoid any contamination. Use aseptic conditions for all transfer, clean bench and surfaces with 70% ethanol.

Day 4.

d. Purification of h5MP1, heIF2$\beta_{53\text{-}136}$ and heIF3c$_{20\text{-}102}$.
i. Thaw bacterial pellets at room temperature and resuspended in 35 mL/1 liter culture binding buffer (this buffer may contain protease inhibitors, see troubleshooting Problem 1). Lyse cells by sonication (10 s on; 20 s off cycle for a total of 3.5 min processing time at 95% amplitude) in ice-water mixed bath.
ii. Centrifuge cell lysate at 40,000 × *g* on a Beckmann JA 30.1 rotor for 40 min at 4°C and collect the supernatant and sterilize through a 0.22 μm filter (Celltreat).

Note: Take 20 μL aliquot of cell lysate before centrifugation, add 5 μL 5× protein loading dye and boil for 2–3 min at 98°C. Label it as whole cell lysate and keep it aside to run on SDS-PAGE later.

iii. Equilibrate Ni-NTA column (5 mL resin bed volume) with binding buffer to at least 5 CV (25 mL). Load clear lysate to the column at 2 mL/min. Collect the flow-through.

Note: Take 20 μL aliquot of flow-through, add 5 μL 5× protein loading dye and boil for 2–3 min at 98°C. Label it as flow through and keep it aside to run on SDS-PAGE later.

iv. Wash the column with binding buffer until the absorbance at 280 reaches a plateau. This is followed by a second wash step with 5% of elution buffer. A peak should elute containing non-specific binding proteins. Wash until absorbance decreases and reaches a plateau. Collect all washes.

Note: Take 20 μL aliquot of washes, add 5 μL 5× protein loading dye and boil for 2–3 min at 98°C. Label it 5% wash and keep it aside to run on SDS-PAGE later.

v. Elute protein in 9 mL fractions with 50% elution buffer.
vi. Analyze eluted fractions along with whole cell lysate, flow-through and washes on 12% SDS-PAGE gel, and pool together the fractions containing purified proteins.

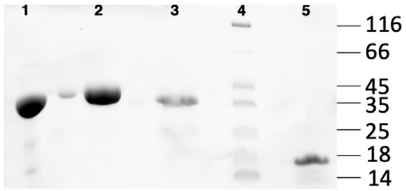


Figure 1. SDS-PAGE of purified proteins used in the ITC experiments

Lane 1: h5MP1-WT; Lane 2: h5MP1-*BN1*; Lane 3: h5MP1-*7A*; Lane 4: Fisher EZ-run markers (116, 66, 45, 35, 25, 18 and 14 KDa); Lane 5 (heIF2$\beta_{53\text{-}136}$, GB1- and His-tags removed).

vii. Remove the His-tag was by adding Thrombin (Hemotech) 10 Units/mg to protein and dialyze overnight at 4°C against Thrombin Dialysis buffer.

Day 5.

viii. Analyze the digestion on a 12% SDS-PAGE. If the tag has been removed, add benzamidine to a final concentration of 1 mM. Load the protein solution onto a Ni-NTA column pre-equilibrated with binding buffer to remove any remanent non-cleaved protein. Collect flow-through.

Note: After His-tag removal, protein does not bind to column and will come in flow through. Still check 5% wash for any non-specific binding of protein to column.

ix. Concentrate protein using Centrifugal Filtration Unit (10 kDa MW Cut off) to 3 mL and load onto a HiLoad 16/60 Superdex 75 preparation-grade column pre-equilibrated with size-exclusion buffer (10 mM HEPES pH 7.5, 125 mM NaCl, 1 mM TCEP) for further polishing and removal of the small amount of thrombin.

x. Analyze the eluted fractions on a 12% SDS PAGE for purity. Pool fractions with protein and concentrate to 2 mg/mL final concentration, aliquote and store at −80°C freezer for the range of duration between 3 to 12 months (depending on protein purification fractions). See Figure 1, lanes 1–3 for the final products.

Note: All buffers for purification need to be prepared with cold deionized water, filter with 0.22 μM filter and store at 4°C. All columns used for purification need to be stored at cold temperature.

Note: All protein purification steps need to be done over ice or at 4°C.

2. Preparation of lysates containing GST-h5MP1.

The lysates containing GST-h5MP1 proteins (WT, 7A or BN1 mutant) for their immobilization to the anti-GST biosensors in BLI were prepared as described in 1) up to step d-ii, except that BL21(DE3) transformants carrying pGEX-h5MP1 or its derivative were IPTG (0.5 mM) -induced for 1 h at 30°C and lysed in PBS containing 1% Triton X-100. Intact full-length GST-fusion product was analyzed as follows:

a. 250 μL of the cell lysates is added to 4 μL bed-volume of Glutathione Sepharose 4B and incubated for 30 min at room temperature on a rocker.
b. Wash the resins three times with 0.2 mL ice-cold PBS.
c. Add 3 μL of 5× Protein Loading Buffer and heat the tubes at 95°C for 2 min to elute the bound GST fusion proteins.
d. Analyze the supernatant by SDS-PAGE, followed by Coomassie Blue staining.

Note: GST-fusion protein concentration is expected to be ~20 μg/mL.

Isothermal titration calorimetry (ITC)

Timing: 1–1.5 h each run

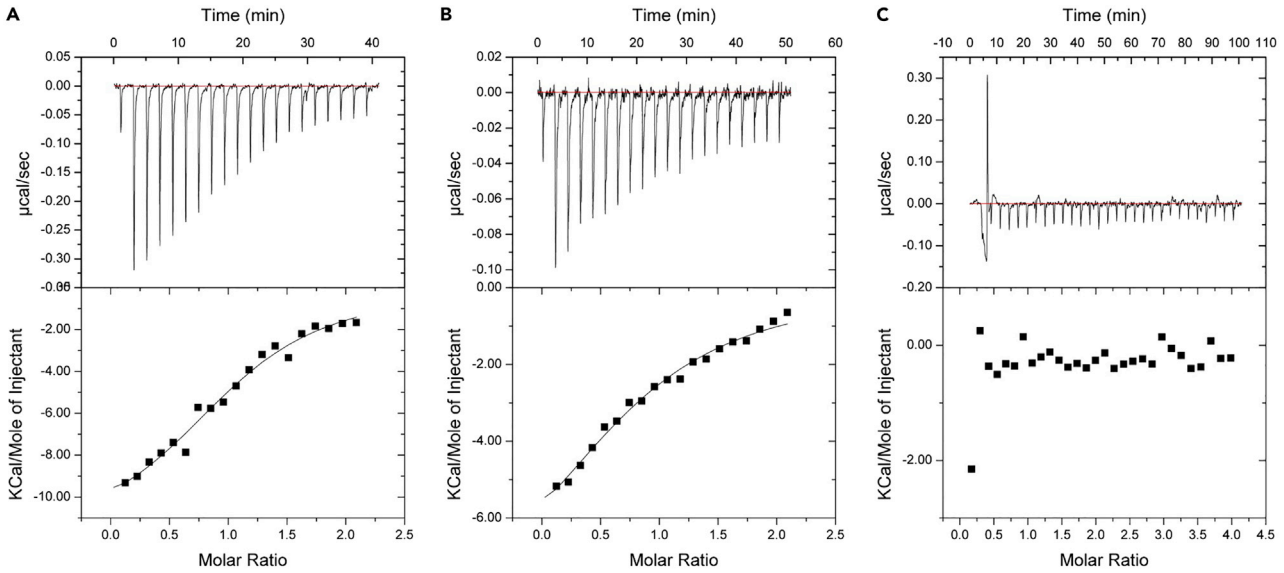


Figure 2. Representative single ITC titrations

The raw data of the titrations is shown in top while the non-linear least square fit of the integrated heats as a function of the molar ratio of the different protein pairs.

(A–C) (A) h5MP1-WT/heIF2$\beta_{53\text{-}136}$; (B) 5MP1-*BN1*/heIF2$\beta_{53\text{-}136}$ and (C) 5MP1-*7A*/heIF2$\beta_{53\text{-}136}$.

Experiments are performed on an MicroCal ITC200 instrument. This assay uses untagged proteins.

3. Setting up ITC run.
   a. All proteins used in the ITC must be buffer exchanged into size exclusion buffer using HiLoad 16/60 Superdex 75 column.
   b. Prepare all solutions of h5MP1 proteins (WT or BN1/7A mutants) to 20 µM final concentration. heIF2$\beta_{53\text{-}136}$ is at 200 µM final concentration.
   c. Wash Cell & Syringe of MicroCal ITC$_{200}$ instrument thoroughly with deionized water followed by size-exclusion buffer.
   d. Fill cell with 200 µL of h5MP1 using a Hamilton syringe making sure no bubbles are left in the chamber by gently agitating the chamber with the syringe.
   e. Fill the pipette/injector with 40 µL of heIF2$\beta_{53\text{-}136}$ using the automated injection fill step. Move pipette/injector into the filled cell chamber.
4. Running ITC titration.
   a. The program will wait for a stable baseline at an equilibrium temperature of 25°C.
   b. The program setting consists of 20 injections with first unique injection of 0.5 µL and remaining 19 injections of 2 µL each with 2n injection time where n=injection volume and 150 s spacing between each injection. The agitation speed was set to 750 rpm).
5. Data Analysis.
   a. Analyze all datasets (Figure 2, top) to obtain the binding constant, stoichiometry and binding enthalpy using the single site model within the ITC data analysis package of the Origin software. The software automatically determines the fitting parameters such as the baseline, the range to integrate the injection peaks and the initialization of the fitting parameters upon calculation of the heat of reactions (Figure 2, bottom).
6. Conclusions.

Repeat experiments under same condition for a total of 3 runs. The ITC200 Origin software calculates all the thermodynamic parameters based on the initial concentrations of the proteins, these included ΔH, ΔG, ΔS, Kd and stoichiometry.

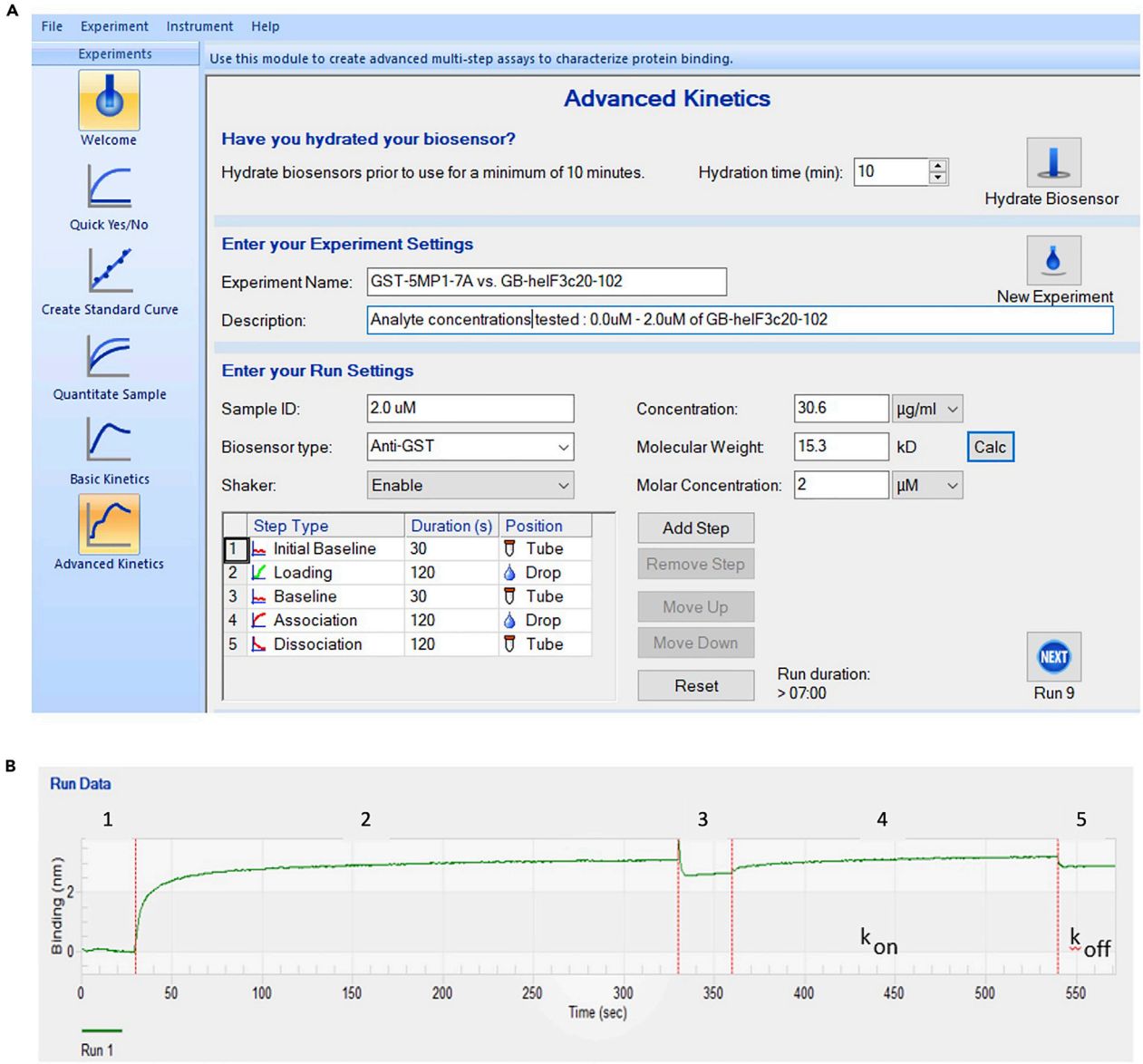


Figure 3. Operation of the BLItz system

(A) Operation interface and parameters.

(B) Chronological steps of operations. 1, Initial baseline; 2, Loading; 3, Baseline; 4, Association; 5. Dissociation.

Biolayer interferometry (BLI)

Timing: 3 h each run

Experiments were performed on a BLItz Label-Free Protein Analysis System from ForteBio. This assay uses GST-5MP1 as ligand; and purified heIF3c$_{20\text{-}102}$ and GB-heIF2β$_{53\text{-}136}$ as analytes.

7. Biosensors activation and setting up the BLItz system (Figure 3A).
   a. Six anti-GST biosensors were first hydrated using 200 µL BLI binding buffer for 10 min in a tube. BSA and Tween-20 were added to avoid any non-specific binding. The biosensors were kept hydrated in PBS buffer until binding experiments were ready.

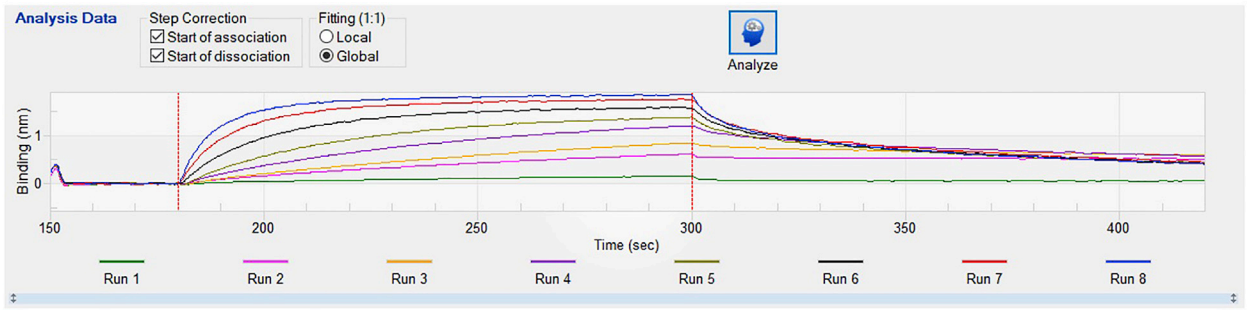
b. Advanced kinetic module was chosen as experiment type to perform the affinity binding assay with 2000 rpm shaker speed. GST-5MP1 was first loaded on the anti-GST biosensor followed by analyte association and dissociation.
c. The parameter for Initial Baseline, Loading, Baseline, Association and Dissociation are set at 30, 120, 30, 120 and 120 s respectively.
d. Bacterial cell lysates containing GST-5MP1, GST-5MP1-BN1 and GST-5MP1-7A proteins were used as ligands to immobilize on the anti-GST biosensors. The two proteins, heIF3c$_{20\text{-}102}$ and GB-heIF2β$_{53\text{-}136}$ purified for ITC experiment were used as analytes. Their concentrations were measured using Protein Bradford Assay and verified with SDS-PAGE. Prior to setting the experiments multiple simple binding assays were performed to check the binding pattern of ligand-analyte interaction.

Note: The initial preliminary runs provide information for deciding the different concentrations to be used in the experiment. Accordingly, different concentrations (0 μM reference, 0.031 μM, 0.0625 μM, 0.125 μM, 0.25 μM, 0.5 μM, 1.0 μM, 2.0 μM) of the analyte were freshly prepared and equilibrated at the room temperature.

Note: We used GST proteins in the lysates instead of purified ones, because the former contains other bacterial proteins which act as good blocking agents on the biosensor. The anti GST biosensor is very specific for GST tagged proteins; even if there are non-specific traces bound, they were cleared up in the subsequent washing step.

8. Ligand binding using Anti-GST biosensor (Figure 3B, steps 7–9).
   a. Conduct Initial Baseline for 30 s prior to the GST-5MP1 or GST-5MP1-BN1 or GST-5MP1-7A loading on the chip using the drop holder.
   b. Perform Loading step using 4 μL lysate of GST tagged proteins. Set the Loading duration for 120 s with shaker speed at 2000 rpm.
   c. Conduct a 30 s baseline step to remove unbound GST-5MP1 proteins using tube position. After every step, wash the drop holder three times using double distilled water.
9. Analytes association and dissociation steps through Advanced Kinetic assay module (Figure 3B, steps 10 and 11).
   a. Perform the Association step for different concentration of the purified analytes, heIF3c$_{20\text{-}102}$ and GB-heIF2β$_{53\text{-}136}$, starting with 0 μM concentration as reference to be incorporated during the data analysis. Set the parameter for this step at 120 s and 2000 rpm and generate an association curve.
   b. Perform the Dissociation step for 120 s at 2000 rpm. Do this measurement using 200 μL of BLI binding buffer to get the dissociation curve.
   c. Repeat the sequential steps of Initial Baseline to Dissociation for each concentration of the GB-heIF3C$_{20\text{-}102}$ or GB-heIF2β$_{53\text{-}136}$ by replacing new Anti-GST biosensors.
   d. Complete 6 runs of an assay for every ligand and analyte combination, during which, the run data are closely monitored.
   e. If the sensogram comes out irregular with erratic curves for various reasons, repeat the step by additional runs. Generally it is caused by tiny air-bubbles introduced while pipetting the solution. Tiny air-bubbles can affect the refractive index and thus the interference pattern. Exclude The while analyzing the data.
10. Analysis of data (Figure 4).
   a. Perform data analysis with the BLItz Pro software. Following the runs, carefully observe every measurement curve for any erratic patterns. After the erratic runs, analyze the data using Global fitting (1:1) to display curve fits and results (Figure 4A).
   b. Enable step corrections for both for the start of association and the start of dissociation; Set the fitting parameter to Global.
   c. Record final results (Figure 4B) and export them into an excel file for additional statistical analysis (e.g., by 2-tailed students' Ttests).

A



B

Index	Sample ID	Conc. (μM)	Information	KD (M)	ka (1/Ms)	ka Error	kd (1/s)	kd Error	Rmax	Rmax Error	R equilibrium	X^2	R^2
1	0 uM	0											
2	0.031 uM	0.031		2.915E-7	5.468E4	1.642E3	1.594E-2	1.887E-4	22.72	1.638	2.184	24.46	0.9437
3	0.0625 uM	0.0625		2.915E-7	5.468E4	1.642E3	1.594E-2	1.887E-4	9.635	0.4133	1.701	24.46	0.9437
4	0.125 uM	0.125		2.915E-7	5.468E4	1.642E3	1.594E-2	1.887E-4	5.728	0.1911	1.719	24.46	0.9437
5	0.25 uM	0.25		2.915E-7	5.468E4	1.642E3	1.594E-2	1.887E-4	3.345	0.08657	1.544	24.46	0.9437
6	0.5 uM	0.5		2.915E-7	5.468E4	1.642E3	1.594E-2	1.887E-4	2.563	0.04761	1.619	24.46	0.9437
7	1.0 uM	1		2.915E-7	5.468E4	1.642E3	1.594E-2	1.887E-4	2.188	0.02668	1.694	24.46	0.9437
8	2.0 uM	2		2.915E-7	5.468E4	1.642E3	1.594E-2	1.887E-4	1.963	0.01589	1.713	24.46	0.9437

Figure 4. The BLItz data analysis
(A) An example of global fitting (1:1).
(B) An example of final results showing deduced kinetic parameters.

11. Conclusion.

Repeat experiments for at least 3 runs for every combination of ligand and analyte. Following the assay analysis, BLItz Pro software could generate association rate constant, k_a (1/Ms), dissociation rate constant, k_d (1/s), and affinity constant, K_D (M). In addition, maximum response (R_{max}), standard error in R_{max} and response at equilibrium ($R_{equilibrium}$) could also be provided.

EXPECTED OUTCOMES

For protein purification, we expect a single protein species with expected molecular weight of >95% purity on SDS-PAGE. If a protein with unexpected size is observed in addition to or in place of the expected product, it is either its degradation product (if shorter than expected) or unrelated co-purifying *E. coli* protein and cannot be used in the downstream experiment (see below troubleshooting).

In ITC, it is expected to obtain dissociation constant (K_D) for the binding affinity and stoichiometry (N) between the two molecules. In BLI, association and dissociation rate constants (k_a and k_d) are obtained besides K_D. However, the interpretation of the obtained values needs careful consideration and examination (see below limitations and troubleshooting).

LIMITATIONS

K_D for interaction between given two molecules can vary widely depending on the methods used to measure it. For example, K_D for interaction between h5MP1 and eIF2$\beta_{53\text{-}136}$ was 0.12 $\pm$ 0.01 μM in BLI and 5.0 $\pm$ 0.15 μM in ITC (Singh et al., 2021). The difference may be due to that in the conformation of interacting molecules, such as due to attachment of different peptide tags (such as GST or GB1) or buffer conditions. Ideally, the most trustworthy values should be obtained when the proteins of interest have a functional conformation as found in vivo. Additionally, the difference may arise due to purely technological reasons. For example, K_D determination by ITC can be easily hindered if one or both of the interacting molecules change(s) conformation, thereby releasing or absorbing heat. In

agreement with this, we were not able to obtain K_D for interaction between 5MP1 and heIF3c$_{20-102}$ in ITC, even though K_D of 0.20 $\pm$ 0.04 μM was obtained for the same interaction in BLI. Given these technical limitations, it is more important to use these methods in mutational analyses to determine the magnitude of the effect of mutations introduced to a protein of interest. Then, the result is carefully compared to that obtained by functional assays in fully or partially reconstituted systems, or ultimately in cells or in vivo. In this way, one can dissect the interaction and thereby determine the physiological relevance of the interaction under the study. Likewise, other parameters obtained by these approaches, such as the stoichiometry number, N, by ITC, must be carefully interpreted in comparison with the results from other functional interaction assays or structural biology approaches (Obayashi et al., 2017).

TROUBLESHOOTING

Problem 1

The yield of purified protein is low.

Potential solution

The protein may be unstable under the growth conditions. The stability of protein is determined by the balance in the rates of its production and degradation. Thus, changes in growth conditions can sometimes lead to changes in protein stability and hence its yield. Typical alternative temperatures to be tested are 30°C and 18°C (if a refrigerated incubator is not available, flasks are shaken in an open platform shaker at the room temperature of 20°C set in a well air-conditioned room). Our favorite low temperature alternative is the growth at 20°C for overnight. To do so, grow bacteria at 37°C till A600=0.5. Then the flask containing the culture is cooled quickly in ice water mixture to ~20°C and begins to be shaken after adding IPTG at a lower concentration (e.g., 0.05 mM). Alternatively, other media such as 2xYT or different concentration of IPTG (as low as 0.05 mM) can be tested. If none of these works, alternatively designed constructs, such as solubilization-enhancement tagging (Reibarkh et al., 2008; Zhou et al., 2001) or mutations to increase protein solubility, or different expression systems (such as baculovirus expression system) must be sought.

Problem 2

There are many other protein bands in purified protein fraction.

Potential solution

Even with the high-resolution preparatory column, you may not be able to separate the protein of interest with co-purifying proteins or its degradation products. In this case, it is advised to change the bacterial growth conditions to minimize expression of the co-purifying protein or suppressing protein degradation. The latter may be alternatively achieved by adding protease inhibitors to the lysis buffer, such as phenylmethanesulfonyl fluoride (PMSF), leupeptin, aprotinin and pepstatin, or simply adding commercially available protease-inhibitor cocktail (Singh and Asano, 2007).

Problem 3

ITC does not detect interaction.

Potential solution

Heat content changes caused by protein conformational changes can compensate for those caused by protein-protein interaction, hence hindering its detection through ITC. Alternatively, the high concentration of the proteins under the experimental conditions may allow them to precipitate, physically blocking their interaction. This can be solved by switching the protein component in the sample cell (if the concentration of the protein in the cell is different from that of the injectant protein), or by increasing the solubility of the proteins through changing buffer conditions or introducing a solubilization-enhancement tag (Zhou et al., 2001) or mutations to increase protein solubility (e.g., by substituting surface-exposed hydrophobic amino acids with polar amino acids).

Problem 4

The K_D values obtained by ITC vary from experiment to experiment.

Potential solution

If the high concentration of the proteins under the experimental conditions causes them to precipitate and the rate of precipitation varies between experiments, this can result in variation in the ITC outputs. Again, this can be solved by increasing the solubility of the proteins through changing buffer conditions or introducing a solubilization-enhancement tag (Zhou et al., 2001) or mutations to increase protein solubility.

Problem 5

The kinetic parameters obtained by BLI vary from experiment to experiment.

Potential solution

In an optical biosensor experiment such as BLI, affinity can be overestimated through avidity effects, i.e., through one dimer (or multimer) binding to two (or more) immobilized molecules. These effects have been documented frequently in surface plasmon resonance (Ladbury et al., 1995). Avidity effects can also influence reproducibility of BLI outputs, as they are computed from a series of titration experiments and as multimer formation may likely depend on concentration of the protein forming multimers. Of the common protein tag added for convenience of purification, GST is known to form dimers (Singh and Asano, 2007). Thus, we avoided avidity effects in our BLI approach through immobilizing GST-h5MP1 fusion into the sensor. By the same token, if a protein under the study is known to form dimer or multimer, one way to avoid/minimize avidity effects and thereby gain reproducible results is to attach this protein to the biosensor chip.

Similar to ITC, values obtained from BLI assays must be interpreted carefully in reference to the results from complementary interaction or functional assays.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Katsura Asano, kasano@ksu.edu.

Materials availability

Plasmids generated in this study are available upon request (materials transfer agreement may be necessary).

This study did not generate new unique reagents.

Data and code availability

The published article (Singh et al., 2021) includes all datasets/codes generated or analyzed during this study.

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AUTHOR CONTRIBUTIONS

C.R.S., R.J., C.E., and K.A. wrote the original draft. K.A. edited and completed the manuscript.

DECLARATION OF INTERESTS

Authors declare no conflict of interests.

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