In-solution Characterization of Biomolecular Interaction Kinetics under Native Conditions

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Keywords: Complex matrix, In-solution, Kinetics, Label free, Protein-protein interactions

ABSTRACT

Characterizing the kinetics of biomolecular interactions is fundamental for understanding biological mechanisms, developing novel drugs, and optimizing processes in protein engineering. Although modern surface-based methods have advanced our understanding of protein-protein and protein-ligand kinetics, they rely on immobilized samples, preventing the study of interactions under native conditions and leading to an incomplete understanding. We propose a paradigm shift by introducing a method based on flow-induced dispersion analysis to study interaction kinetics while keeping biomolecules in solution, eliminating the need for surface immobilization and ensuring truly native binding conditions. The method examines reactions outside equilibrium by inducing a rapid concentration change in one of the binding partners (C-Jump) in a controlled microfluidic environment. Notably, it operates without buffer restrictions and requires minimal sample quantities. We demonstrate C-Jump's capability by accurately determining the association and dissociation rates of protein-protein and protein-small molecule interactions. Furthermore, we validate its robustness by measuring a protein-protein interaction in human serum and a protein-small molecule interaction in-solution, label-free. This underlines C-Jump's broad applicability for studying biomolecular interactions under true native conditions, offering a powerful tool for protein engineering and drug discovery, as well as enabling characterization of previously inaccessible interactions.

INTRODUCTION

The ability to accurately quantify biomolecular interactions is fundamental to fields ranging from biophysics and structural biology to drug discovery and protein engineering. Kinetic measurements of protein-protein and protein-ligand interactions provide essential insights into molecular recognition, enzymatic activity, and therapeutic efficacy.[1–3] Over the past decades, surface-based techniques such as surface plasmon resonance (SPR) and bio-layer interferometry (BLI) have emerged as gold standards to quantify kinetic parameters.[4,5] However, these approaches inherently rely on immobilized binding partners, introducing potential artifacts due to surface effects, steric hindrance, and diffusion limitations.[6] Consequently, the observed kinetics may not fully reflect interactions in solution, where biological processes naturally occur. Here, we introduce C-Jump, a method based on flow induced dispersion analysis (FIDA)[7,8], that eliminates the need for surface immobilization, operates without buffer constraints, and requires only minimal amount of protein sample for truly native conditions. In brief, a small volume of protein sample is pulse-injected into a narrow capillary containing its binding partner. A laminar flow rapidly mixes and dilutes the sample with its binding partner inducing a concentration jump.[9]

By changing the flow velocity, the time to detection can be adjusted and, consequently, the reaction time. This allows to study the interaction before equilibrium is reached. A series of measurements at different ligand concentrations creates a binding curve outside of equilibrium, allowing precise determination of both the association rate (k_{on}) and dissociation rate (k_{off}).

To demonstrate C-Jump and its versatility, we apply it to measure both protein-protein and protein-small molecule interactions in solution, showing that it yields highly accurate association and dissociation rates. Furthermore, we validate its robustness with a kinetic measurement in human serum and a label-free measurement, closely mimicking true native conditions. By eliminating the constraints of immobilization-based methods, C-Jump enables kinetic analyses in solution, unlocking interactions previously inaccessible to study. Its broad applicability, ease of implementation, and compatibility with complex biological samples makes it a versatile tool for research at the interface of biophysics, chemistry, and medicine.

RESULTS AND DISCUSSION

A non-covalent interaction between a target and a ligand (termed indicator and analyte, respectively) is governed by its association rate (k_{on}) and dissociation rate (k_{off}). At equilibrium, the ratio (k_{off}/k_{on}) defines the dissociation constant (K_d), which characterizes the overall affinity between interaction partners. Here we present C-Jump, a method that enables precise kinetic measurements by inducing rapid concentration changes in a controlled microfluidic environment. We first outline its theoretical framework before demonstrating its applicability to three well-characterized model systems. Finally, we showcase its capability to measure in-solution kinetics of a protein-protein interaction in human serum and a label-free protein-small molecule interaction.

C-Jump. To measure the reaction rates with FIDA under Taylor conditions[10], two binding curves must be obtained: one at equilibrium and one outside equilibrium (Figure 1A). While equilibrium measurements are obtained from a preincubated mixture of the interaction partners (Figure1 B, top), interactions outside equilibrium are captured using the capillary mixing method (CapMix)[11] which allows a precise control of the interaction time. In brief, a small volume of the indicator is injected between two zones of analyte and a pressure-induced flow rapidly mixes the components in the capillary (Figure1 B, bottom). This results in a rapid change of concentration (C-Jump).[9] In a CapMix experiment the reaction begins upon applying the run pressure p_{run}. The reaction time coincides with the reference time (t_R), which is the time it takes for the sample to reach the point of detection and is measured using the peak of the recorded fluorescence signal. By increasing p_{run}, the time to detection – and thus the reaction time – can be significantly reduced

(Figure1 C), allowing measurements to be taken before equilibrium is reached. To ensure measurements remain within Taylor conditions, the capillary diameter and length can be adjusted accordingly. Under these conditions, the apparent hydrodynamic radius (R_h) reflects the weighted average between the diffusivity of the unbound indicator and the indicator-analyte complex.[7] Thus, the shorter the interaction time, the smaller the apparent R_h. Crucially, this dependence of R_h on the interaction time provides direct kinetic insights. To quantitatively describe this relationship, we present an analytical expression that directly links the observed hydrodynamic radius to K_d, as well as k_{on} and k_{off} (Equation S16 in the supporting information). Furthermore, we extend this approach to ratiometric fluorescence[12] as an additional readout for systems where the analyte is a small molecule compared to the indicator and hence, size changes are difficult to detect (Equation S23 in the supporting information).[13] These expressions enable precise extraction of kinetic parameters from experimental data, allowing robust characterization of biomolecular interactions in solution under native conditions.

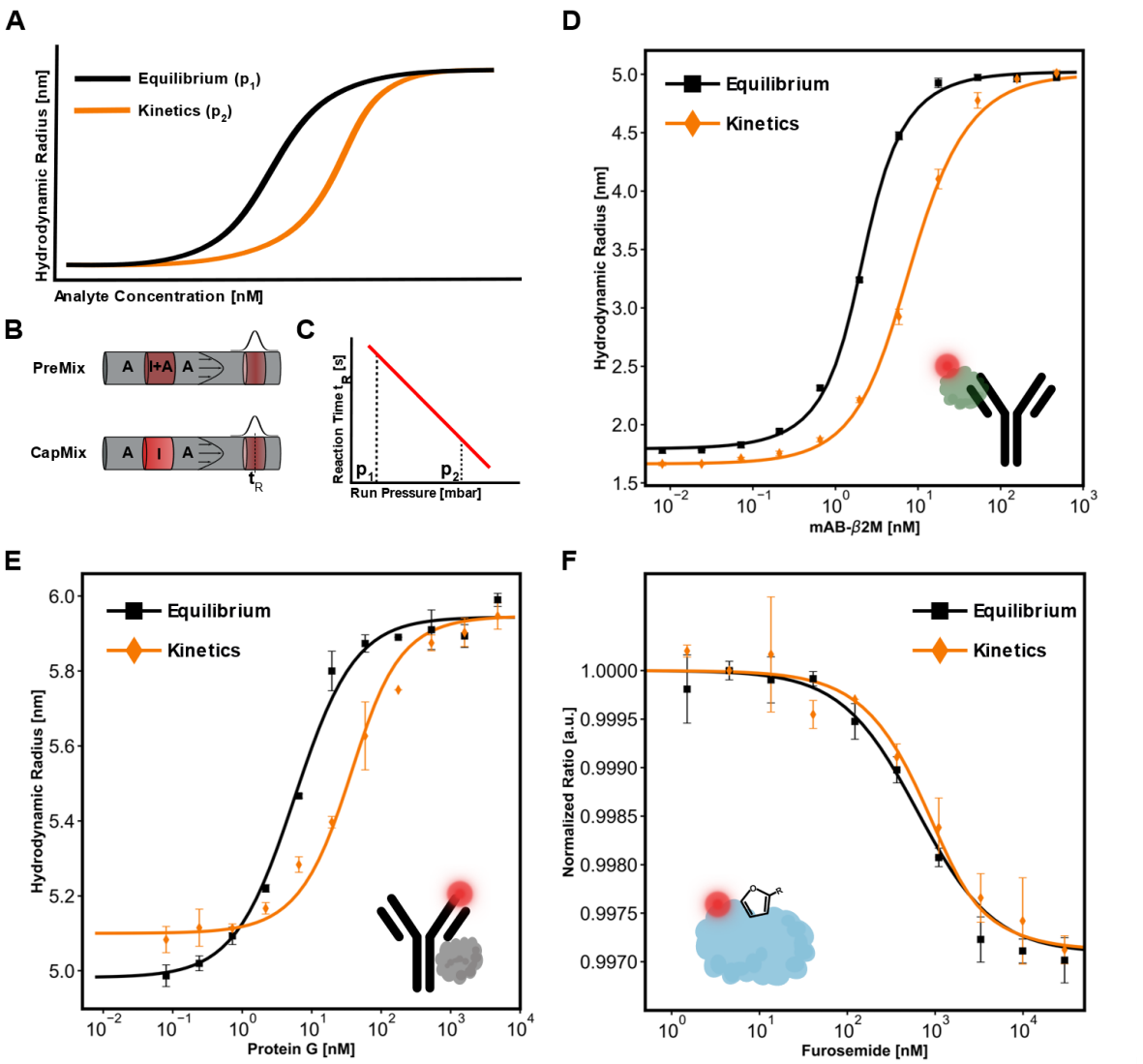

Figure 1. Theoretical outline and proof of concept for C-Jump. ***(A)*** *Sketch of binding curves under equilibrium conditions (black) and kinetic conditions (orange). Near the K_d, the same analyte concentration results in a smaller apparent R_h.* ***(B)*** *Experimental layout for PreMix and CapMix methods to study interactions at and out of equilibrium.* ***(C)*** *Linear relationship between run pressure (p_{run}) and reaction time (t_R).* ***(D-F)*** *Measured equilibrium and kinetic binding curves for specific interactions. Each data point represents the mean of three independent measurements, with error bars indicating the standard error of the mean.* ***(D)*** *β2M* + mAb-β2M,* ***(E)*** *Rituximab* + Protein G, and* ***(F)*** *CAII* + FS. The asterix (*) marks the indicator labeled with the dye ALC647, also illustrated in the molecule sketches with a red fluorophore.*

Proof of principle. To validate C-Jump, we selected three well-characterized biomolecular interactions previously studied using surface-based methods. By comparing C-Jump data to established equilibrium and kinetic parameters, we assessed its accuracy and reliability. The resulting binding curves are shown in Figure 1D-F, with the equilibrium binding curves in black and the kinetics curves in orange. Each data point represents the mean of three independent

measurements, with error bars indicating the standard error of the mean. The schematics in the corners of the graphs illustrate the respective interactions, with a red fluorophore marking the labeled indicator.

Figure 1D presents the binding curves for the protein-protein interactions of β2-Microglobulin (β2M) with its corresponding monoclonal antibody (mAB-β2M) and Figure 1E shows Rituximab with Protein G. Both interactions exhibit a measurable increase in hydrodynamic radius upon binding, with a distinct rightward shift of the kinetic curve relative to the equilibrium curve. In both cases, one of the binding partners is an antibody, with Rituximab serving as the labeled indicator and mAb-β2M as the analyte in the respective interactions. In the case of Rituximab, the observed R_h of the unbound indicator corresponds to the size of an IgG antibody (~150 kDa), whereas for β2M, the complex size also aligns with that of an IgG due to the much smaller size of β2M. Figure 1F shows the normalized binding curves for the interaction between carbonic anhydrase II (CAII) and furosemide (FS), where changes in fluorescence ratio were used to resolve binding. Despite the small relative fluorescence change (0.5 %), the binding curves remain well-defined, demonstrating the sensitivity of the detection method. The extracted interaction parameters are summarized in Table 1, alongside values obtained from surface-based measurements reported in the literature. Minor variations, such as those observed in the CAII–FS interaction, can be attributed to differences in buffer conditions and batch-to-batch variability of the proteins. However, more substantial discrepancies, as seen in the Rituximab–Protein G interaction, are particularly evident in the dissociation rate, which is an order of magnitude faster in solution, leading to a higher K_d. This discrepancy suggests that immobilization in surface-based assays may stabilize the complex, possibly due to avidity effects, thereby reducing its off-rate. For the interaction between β2M and mAB-β2M, the in-solution method yields a similar dissociation rate to the surface-based approach but reveals an association rate that is an order of magnitude faster. This substantial increase likely arises from the greater conformational freedom in solution, allowing the proteins to adopt favorable orientations for binding, in contrast to the restricted geometry that may be imposed by surface immobilization. Additionally, different immobilization strategies can influence affinity in surface-based assays, as demonstrated for β2M, where a four-fold change in K_d and two-fold variations in kinetic rates have been reported.[14] Such effects highlight the importance of measuring interactions in solution, where proteins remain in their true native state without potential structural constraints from surface attachment.

Table 1. Binding affinities and kinetic rate constants measured in-solution with C-Jump and reported values for surface-based assays (asterix (*) indicates the labeled species).

	β2M* - mAB-β2M		Rituximab* – Protein G		CAII* - FS	
	C-Jump	SPR	C-Jump	SPR	C-Jump	SPR
K_d [nM]	0.32 ± 0.05	1.0 [14]	4.7 ± 0.6	0.9[15]	555 ± 80	330 [16]
k_{on} [$s^{-1}M^{-1}$]	$(1.2 \pm 0.1) \times 10^7$	1.6×10^6 [14]	$(2.8 \pm 0.5) \times 10^5$	1.9×10^5[15]	$(5.4 \pm 1.6) \times 10^4$	9.6×10^4 [16]
k_{off} [s^{-1}]	$(4.0 \pm 0.2) \times 10^{-3}$	1.6×10^{-3}[14]	$(1.2 \pm 0.2) \times 10^{-3}$	1.7×10^{-4}[15]	$(3.0 \pm 0.9) \times 10^{-2}$	5.0×10^{-2} [16]

Application. We further demonstrate the capabilities of C-Jump by applying the method to study the interaction between Interleukin-2 (IL-2) and its receptor Interleukin-2Rα (IL-2Rα) under physiologically relevant conditions in 90 % human serum. This environment, which closely mimics in vivo conditions, is challenging conventional surface-based methods due to nonspecific adsorption and signal interference.[17] To accommodate the expected fast kinetics of the interaction[18], the experiment was performed in a shorter capillary at 15 °C. Figure 2A presents the resulting binding curves, yielding an association rate of $k_{on} = (1.9 \pm 0.8) \times 10^6\ \mathrm{M^{-1}s^{-1}}$ and a dissociation rate of $k_{off} = 0.04 \pm 0.01\ \mathrm{s^{-1}}$, revealing faster kinetics compared to phosphate-buffered saline (PBS) at the same temperature (Figure S2A). Notably, the association and dissociation rates in serum at 15 °C closely matched those measured in PBS at 25 °C (Table S1), as well as previously reported values from a surface-based assay.[18] A potential explanation for this acceleration is a structural rearrangement of IL-2 in serum, as indicated by the significantly larger apparent R_h of both the indicator and the complex compared to their sizes in PBS (Figure S2). Similar serum-induced structural changes have been reported for other proteins[19] and may expose the binding pocket, facilitating faster binding and unbinding events. Interestingly, direct measurements in serum at 25 °C were not feasible, as the rapid kinetics exceeded the experimental detection limit. However, given that the kinetics in serum at 15 °C align with those in PBS at 25 °C, it is likely that the interaction in serum at 25 °C follows a similar trend. These findings emphasize the importance of performing kinetic measurements in truly native biological environments, as serum-specific structural effects can significantly influence interaction dynamics, which may be overlooked in simplified buffer conditions.

To further demonstrate the potential of C-Jump, we performed a label-free, in-solution measurement of the interaction between CAII and FS. We leveraged the ratiometric fluorescence of tryptophan residues (Supporting Information S5), which are highly sensitive to their local environment.[20,21] As shown in Figure 2B, the resulting binding curves yield an association rate of $k_{on} = (1.8 \pm 0.2) \times 10^5\ \mathrm{M^{-1}s^{-1}}$ and a dissociation rate of $k_{off} = 0.07 \pm 0.01\ \mathrm{s^{-1}}$, representing a twofold increase compared to the values reported in Table 1. Notably, the fluorescence ratio

exhibited a remarkable 900 % relative change, corresponding to an 1800-fold increase in sensitivity compared to the labeled experiment. This highlights the exceptional sensitivity of the detection method and demonstrates the potential of C-Jump for studying proteins in their native state.

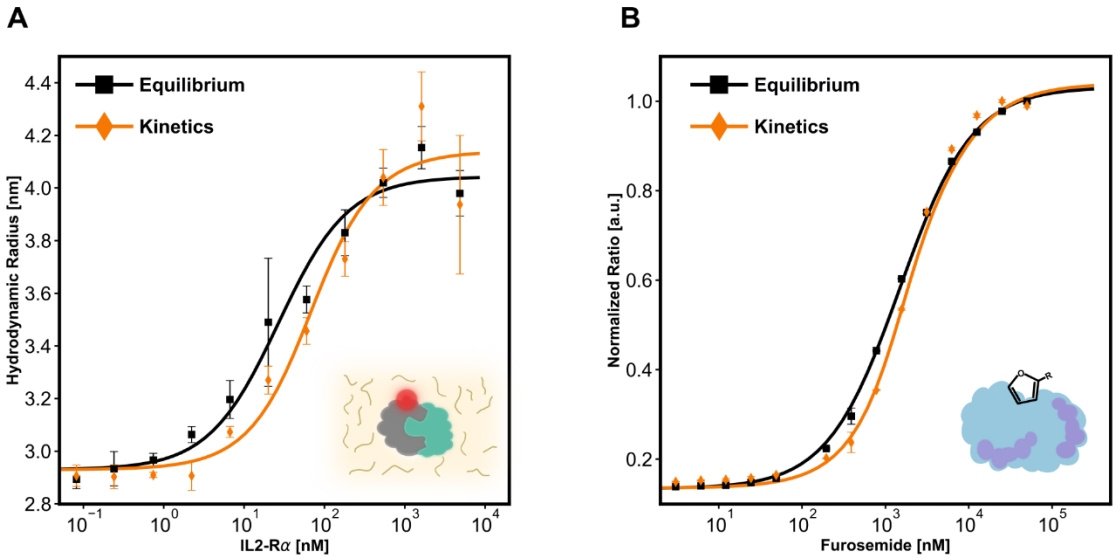

Figure 2. Measured equilibrium and kinetic binding curves in human serum and label-free. Each data point represents the mean of three independent measurements, with error bars indicating the standard error of the mean. ***(A)*** *Kinetics of IL-2 and its receptor IL-2Rα in 90 % human serum. The K_d is 19 nM with the rate constants $k_{on} = (1.9 \pm 0.8) \times 10^6\ M^{-1}s^{-1}$ and $k_{off} = 0.04 \pm 0.01\ s^{-1}$. The apparent R_h of the unbound indicator and the complex are significantly bigger than measured in pure buffer (Figure S2).* ***(B)*** *Kinetics of CAII binding to FS, measured in solution and label-free using ratiometric fluorescence of tryptophan residues. The K_d is 402 nM with the rate constants $k_{on} = (1.9 \pm 0.2) \times 10^5\ M^{-1}s^{-1}$ and $k_{off} = 0.07 \pm 0.01\ s^{-1}$.*

CONCLUSION

Accurate quantification of biomolecular interactions is essential across a broad spectrum of disciplines, including biophysics, structural biology, drug discovery, and protein engineering. Although numerous methods exist to determine binding affinity, the measurement of kinetic rates is predominantly limited to surface-based techniques, which rely on immobilized binding partners and may not accurately mimicking physiological conditions. In this work, we introduce C-Jump, a paradigm shift that directly measures kinetic rates in solution, more closely reflecting the native environment where binding typically occurs. Leveraging flow-induced dispersion analysis, C-Jump is free from buffer constraints, can operate label-free and requires only nanograms of protein. These unique features make C-Jump a versatile and efficient approach,

191 positioning it as a transformative tool for advancing research in drug discovery, biophysics,
192 chemistry, and medicine.

193 **MATERIALS & METHODS**

194 **Equipment.** The experiments were conducted using a Fida Neo 640 system (Catalog No. N002-
195 640, Fida Biosystems ApS) and a Fida Neo UV detector (Catalog No. Det-UVN, Fida Biosystems
196 ApS). Experiments utilize two types of fused silica capillaries (Ø 75 µm, L = 100 cm, Catalog No.
197 100-001 and Ø 50 µm, L = 70 cm, Catalog No. 100-057, Fida Biosystems ApS). Capillaries were
198 pre-treated by flushing with a high-sensitivity coating reagent (Catalog No. 310-010, Fida
199 Biosystems ApS) prior to each experiment.

200 **Materials and Chemicals.** Human β2-Microglobulin (B2M-H5225, Lot 475-2391F1-1LQ), human
201 Interleukin-2 (IL2-H5215) and human Interleukin-2Ralpha (ILA-H52H9) were purchased from
202 Acro biosystems. Anti-β2-Microglobulin monoclonal antibody was sourced from Cytiva
203 (28980886). Bovine carbonic anhydrase II (C2522-1G, Lot No. SLCQ8559) and furosemide (F4381,
204 Lot No. MKCM6678) purchased from Sigma-Aldrich. Rituximab (MA5-47773) and Protein G
205 (101201) were purchased from Thermo Fisher. Phosphate-buffered saline (PBS) 1x was prepared
206 from a 10x PBS stock solution (Lot No. Q01I511, Thermo Scientific) using deionized water (Direct-
207 Q3, Sigma-Aldrich). Additional reagents included human serum type AB (male, H4522-20ML,
208 Sigma-Aldrich) and sodium hydroxide 1 M (NaOH) from sodium hydroxide pellets (Lot No.
209 SLCC5273, Sigma-Aldrich).

210 **Assay buffer.** The assay buffer for all binding studies is PBS (1x, pH 7.4) supplemented with
211 0.03 % Pluronic F-127 (Millipore, Lot No. 3951203).

212 **Protein Labeling.** The storage buffer for β2-Microglobulin (β2M) was exchanged with PBS (1x,
213 pH 7.4) using a spin column (Catalog No. 89882, Thermo Scientific), which was pre-rinsed three
214 times with PBS at 1.5 x 1000 rpm for 60 seconds per rinse. After buffer exchange, the protein
215 concentration was adjusted to 1 mg/mL. Carbonic anhydrase II (CAII) was dissolved in PBS to
216 final concentrations of 1 mg/mL. Lyophilized Interleukin-2 (IL-2) was dissolved in deionized water
217 to a concentration of 1 mg/ml. Proteins were labeled individually using the Fida 1 Protein Labeling
218 Kit ALC 640 (Catalog No. 430-003, Fida Biosystems ApS) by modifying lysine residues with an
219 environmentally sensitive dye. A 1 M sodium bicarbonate buffer was added, followed by the dye
220 at a fourfold molar excess. Incubation occurred in the dark for 30 minutes, and excess dye was
221 removed using a spin column provided in the kit. Degrees of labelling were 1.8 for β2M, 1.4 for
222 CAII, 0.9 for Rituximab and 0.1 for IL-2 (NanoDrop, Thermo Fisher). Free dye content for all

223 proteins was less than 5 % measured with a Fida Neo system 640. For the measurement in human
224 serum, any remaining free dye was removed before the experiment with a spin column provided
225 in the labelling kit. Labeled proteins were stored at -20 °C.

226 **Ligand Preparation.** Anti-β2-Microglobulin monoclonal antibody (mAB-β2M) was received at a
227 stock concentration of 1 mg/mL and stored at 4 °C. Stock solution of the CAII inhibitor furosemide
228 (FS) was prepared in DMSO at 100 mM and stored at 4 °C. Protein G and Interleukin-2Ralpha (IL-
229 2Rα) were prepared from lyophilized powder by adding deionized water to a concentration of
230 1 mg/mL and stored at -20 °C.

231 **Premix Experiment**: Stock solutions of the ligand were diluted in PBS or serum to the following
232 concentrations: 1 µM for mAB-β2M, 10 µM for Protein G, 50 µM for FS, and 10 µM for IL-2Rα. A 12-
233 point dilution series (3-fold dilution) was prepared for each analyte in the respective buffers. A
234 portion of the analyte was transferred and premixed with the corresponding indicator to achieve
235 a final volume of approximately 20 µL. The indicator concentrations for the labeled proteins were
236 10 nM for β2M, 10 nM for Rituximab, 100 nM for CAII, and 30 nM for IL-2. The indicator
237 concentration for the unlabeled CAII was 600 nM.

238 Unless stated otherwise in the text, all experiments were performed at 25 °C. Each data point
239 required 40 nL of protein and 12 µL of analyte and took approx. 5 min. The experimental sequence
240 included: (1) flushing with NaOH at 3500 mbar for 20 s; (2) flushing with deionized water at 3500
241 mbar for 20 s; (3) flushing with coating reagent at 3500 mbar for 20 s. (4) flushing with deionized
242 water at 3500 mbar for 20 s. (5) filling the capillary with analyte solution at 3500 mbar for 20 s; (6)
243 injecting premixed-indicator at 50 mbar for 10 s; (7) mobilizing and measuring with analyte
244 solution at 400 mbar for 180 s. The signal in both spectral channels was recorded simultaneously
245 for the measurement time during step 7.

246 **Kinetics Experiment.** The kinetics experiments were performed immediately following the
247 premix experiment, using the same dilution series of the analyte. The indicator concentration was
248 identical to that used in the premix and was prepared separately in the experimental buffer. For
249 the serum experiment, the indicator was kept in PBS to prevent degradation during the
250 experiment.

251 The procedure for step 1) to 5) is equal to the equilibrium experiment. (6) injecting unbound
252 indicator at 50 mbar for 10 s; (7) mobilizing and measuring with analyte solution at pressures and
253 times as shown in Table 2, including also the used capillary in diameter x length. The signal in
254 both spectral channels was recorded simultaneously for the measurement time during step 7.

255 *Table 2. Conditions for kinetics experiments.*

Interaction	Run Pressure [mbar]	Time [s]	Capillary [µm x m]
β2M & mAB-β2M	1000	50	Ø75 x L1
Rituximab & Prot. G	400	60	Ø75 x L1
CAII & FS	1600	45	Ø75 x L1
CAII & FS (UV)	800	45	Ø75 x L0.7
IL-2 & IL-2Rα	3000	60	Ø50 x L0.7

256

257 **Data Processing.** Data analysis was performed using FIDA Analysis Software (Version 3.1.1.0,
258 Fida Biosystems ApS). Multi-species Gaussian fitting was applied to distinguish between the
259 signal from the labeled protein and the free fluorophore. The extracted peak width of the labeled
260 protein was used to determine its apparent R_h.

261 For ratiometric fluorescence measurements, the fitting procedure was applied to both spectral
262 channels, with the area under the curve representing the total fluorescence. The ratio was then
263 calculated by dividing the total fluorescence of the longer-wavelength spectral channel by that of
264 the shorter-wavelength spectral channel. The mean and standard error of the mean were plotted
265 against the analyte concentration. Binding isotherms were then fitted to obtain dissociation
266 constants, while kinetic experiments provided reaction rate constants. Detailed descriptions of
267 the applied models can be found in the supporting information (Equations S16 and S23).

268 **ASSOCIATED CONTENT**

269 **Supporting Information**. Additional experimental details, data analysis, materials and methods,
270 including schematics of experimental setup (PDF).

271

272 **AUTHOR INFORMATION**

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275 **Acknowledgement.** Financial support from the Innovation Fund Denmark (grant no. 2052-
276 00002B) is gratefully acknowledged.

277 **Conflict of Interest.** The authors declare the following competing financial interest(s): HJ, AH,
278 KRS, ES, and PW have commercial interests in FIDA Biosystems ApS. HJ, AH, KRS, ES, and PW are
279 employees of FIDA Biosystems ApS.

280

References

1. Vauquelin, G. Effects of target binding kinetics on in vivo drug efficacy: koff, kon and rebinding. *Br. J. Pharmacol.* **173**, 2319–2334 (2016).
2. Frieden, C. Protein-protein interaction and enzymatic activity. *Annu. Rev. Biochem.* **40**, 653–696 (1971).
3. Janin, J. L. The Kinetics of Protein-Protein Recognition. *Proteins* 153–161 (1998) doi:10.1002/(SICI)1097-0134(199706)28:2.
4. Abdiche, Y., Malashock, D., Pinkerton, A. & Pons, J. Determining kinetics and affinities of protein interactions using a parallel real-time label-free biosensor, the Octet. *Anal. Biochem.* **377**, 209–217 (2008).
5. Homola, J. Present and future of surface plasmon resonance biosensors. *Anal. Bioanal. Chem.* vol. 377 528–539 Preprint at https://doi.org/10.1007/s00216-003-2101-0 (2003).
6. Schuck, P. & Zhao, H. The Role of Mass Transport Limitation and Surface Heterogeneity in the Biophysical Characterization of Macromolecular Binding Processes by SPR Biosensing. *Methods. Mol. Biol.* **627**, 15 (2010).
7. Jensen, H. & Østergaard, J. Flow induced dispersion analysis quantifies noncovalent interactions in nanoliter samples. *J. Am. Chem. Soc.* **132**, 4070–4071 (2010).
8. Pedersen, M. E., Østergaard, J. & Jensen, H. Flow-induced dispersion analysis (FIDA) for protein quantification and characterization. in *Methods. Mol. Biol.* vol. 1972 109–123 (Humana Press Inc., 2019).
9. Norrild, R. K. *et al.* Taylor Dispersion-Induced Phase Separation for the Efficient Characterisation of Protein Condensate Formation. *Angew. Chem. – Int. Ed.* **63**, (2024).
10. Geoffrey Ingram Taylor. Dispersion of soluble matter in solvent flowing slowly through a tube. *Royal Society* **219**, (1953).
11. Pedersen, M. E., Østergaard, J. & Jensen, H. Quantification of Structural Integrity and Stability Using Nanograms of Protein by Flow-Induced Dispersion Analysis. *Molecules* **27**, (2022).
12. Demchenko, A. P. The concept of λ-ratiometry in fluorescence sensing and imaging. *J. Fluoresc.* vol. 20 1099–1128 Preprint at https://doi.org/10.1007/s10895-010-0644-y (2010).
13. Willmer, P., Hundahl, A. C., Marie, R. & Jensen, H. Continuous Titration Based Method for Rapid In-Solution Analysis of Non-Covalent Interactions. *Chem. Methods* (2025) doi:10.1002/cmtd.202400059.

14. Zhao, H., Gorshkova, I. I., Fu, G. L. & Schuck, P. A comparison of binding surfaces for SPR biosensing using an antibody–antigen system and affinity distribution analysis. *Methods* **59**, 328–335 (2013).

15. Stern, D., Dettmann, P., Dorner, B. G. & Mages, H. W. Protein G affinity chromatography is an underrated but very potent purification method for a broad range of species-independent and tag-less Fab-fragments. *J Immunol Methods* **529**, (2024).

16. Myszka, D. G. Analysis of small-molecule interactions using Biacore S51 technology. *Anal. Biochem.* **329**, 316–323 (2004).

17. Mariani, S. & Minunni, M. Surface plasmon resonance applications in clinical analysis. *Anal. Bioanal. Chem.* **406**, 2303–2323 (2014).

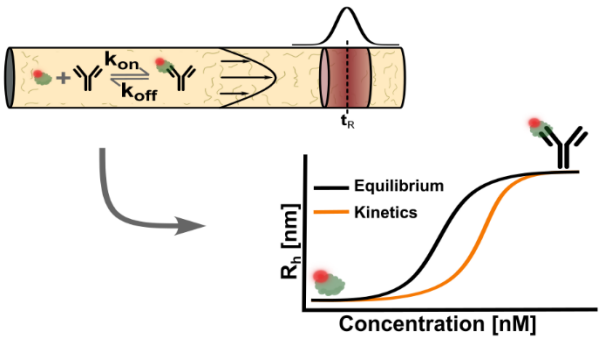
18. Myszka, D. G. *et al.* Kinetic analysis of ligand binding to interleukin-2 receptor complexes created on an optical biosensor surface. *Protein Sci.* **5**, 2468–2478 (1996).

19. Hill, J. J. & Laue, T. M. Protein Assembly in Serum and the Differences from Assembly in Buffer. *Methods. Enzymol.* **562**, 501–527 (2015).

20. Lakowicz, J. R. *Principles of Fluorescence Spectroscopy*. *Principles of Fluorescence Spectroscopy* (Springer US, 1983). doi:10.1007/978-1-4615-7658-7.

21. Valeur, Bernard. *Molecular Fluorescence : Principles and Applications*. (Wiley-VCH, 2002)

TABLE OF CONTENT



This work presents C-Jump, a breakthrough method for the direct measurement of kinetic rates in solution, closely mimicking physiological conditions. Utilizing flow-induced dispersion analysis, C-Jump determines rate constants with high accuracy for protein–protein and protein–small molecule interactions. Free from buffer constraints and requiring only nanograms of protein, it can operate label-free, transforming biomolecular interaction studies across biophysics, chemistry, and medicine.