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Christophe Velours, Magali Aumont-Nicaise, Stephan Uebel, Patrick England, Adrian Velazquez-Campoy, David Stroebel, Guillaume Bec, Pierre Soule, Christophe Quétard, Christine Ebel, et al.

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# European Biophysics Journal

## Macromolecular interactions in vitro, comparing classical and novel approaches --Manuscript Draft--

<b>Manuscript Number:</b>	EBJO-D-20-00187R1	
<b>Full Title:</b>	Macromolecular interactions in vitro, comparing classical and novel approaches	
<b>Article Type:</b>	S.I. : COST Action CA15126, MOBIEU: Between atom and cell	
<b>Keywords:</b>	Molecular scale biophysics; macromolecular interactions; artificial binders; double-stranded DNA breaks repair factors	
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	French Infrastructure for Integrated Structural Biology (ANR-10-INBS-05)	Mr Jean-Baptiste Charbonnier
<b>Abstract:</b>	Biophysical quantification of protein interactions is central to unveil molecular mechanisms of cellular processes. Researchers can choose from a wide panel of biophysical methods, including classical and more novel ones. A real-life proof-of-concept was carried out during an ARBRE-MOBIEU training school held in June 2019 in Gif-sur-Yvette, France. Twenty European students benefited from a one-week	

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<p><b>Response to Reviewers:</b></p>	<p>Reviewer #1: We have incorporated all of your suggestions into our revised manuscript they were very helpful. You will find below the answer to your specific comments:</p> <p>1)Despite the interest of some of the drawn conclusions—and their indubitable didactical value—, the strongly promotional content of the manuscript should be tempered. For instance, Figure S1 should be removed from the paper—maybe it can be substituted for a link to the website of the course in the main text.</p> <p>As required Figure S1 has been removed and substituted by a link to the website of the course in the main text.</p> <p>2)The need of in vitro validation of demonstrated interactions in cell is suggested in two occasions (lines 48 and 630). However, the in cell detection of an interaction constitutes a stronger evidence of its specificity. It would be more adequate to talk about in vitro characterisation, which provides structural, physical and thermodynamical details that cannot be obtained in cell (especially in a quantitative way) and are also important to understand macromolecular interactions.</p> <p>Now lines 49 and 688 have been corrected to describe in vitro characterization instead of validation of macromolecular interactions observed in cell.</p> <p>3)More detailed explanations about the followed procedures in "Materials and methods" section would be desirable rather than general descriptions of the used techniques, so the exposed experiments can be easily reproduced. Similarly, "Samples preparation" subsection barely contains information about experimental procedures.</p> <p>We agree with the reviewer that more detailed explanations about the procedures are missing. A few important details have been included in the revised version.</p> <p>4)Line 124 states that only the interaction between A3 and Rep17 has been studied due to its stronger character versus the one between A3 and Rep2. However, measurements on the A3-Rep2 complex are commented (312). This should be clarified.</p> <p>Now line 134 as required has been clarified in the text, both A3/Rep2 and A3/Rep17 interactions were measured by different methods during the preparation of the training. We chose to measure only the latter during the training because of its higher affinity.</p> <p>5)Regarding binding experiments with labelled AlphaReps, some aspects about methodology and the results should be clarified.</p> <p>We clarified the following questions regarding coupling or labelled A3:</p> <ul style="list-style-type: none"> <li>- It seems plausible that the coupling of A3 to the DNA strand for switchSENSE (line 364) could affect the A3 binding with Rep2, positive or negatively. Has it been tested?</li> <li>-Now line 415: Binding of coupled A3-DNA to Rep2 was tested during the preparation of the training. The measured affinity was comparable with the values obtained previously with other methods, i.e. ITC, SPR (Léger et al., 2019).</li> <li>- Authors state that the used dye for MST experiments substantially improves the signal detection and protein integrity versus the one used in AUC-FDS (line 400). Then, does the latter produce protein instability? In this case, could the His-Tag Red-tris-NTA labelling used for both techniques?</li> <li>-Now line 448: His-Tag Red-tris-NTA labelling cannot be used for AUC-FDS</li> </ul>

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-Upon closer inspection we would like to omit the data set on A3-Rep17 from the blue channel, this measurement does not fulfil the quality criteria applicable to MST data (S/N) and the measured KD cannot be trust it under these conditions.

6)Regarding measurements on the Ku-DNA interaction, some points about the obtained results should be clarified.

We clarified the following points regarding Ku-DNA interactions:

- Authors suggest that SEC-MALS provide peaks corresponding to Ku binding to a 42 bp DNA with both 2:1 and 1:1 stoichiometry (lines 452-459). However, in consistency with the statement of that a 42 bp DNA should bind 2 Ku heterodimers (lines 144-146), a 1:1 binding should be barely or not observed. Could this result be explained by the use of an insufficient Ku concentration (not saturating) for the complexation reaction prior to SEC?

-Now lines 153-155/lines 495-504: SEC-MALS experiments were carried out with an excess of DNA, which corresponded to the last elution peak of the chromatograms. Because, we used a sub-saturating Ku concentration, we observed two types of complexes with the 42bp DNA, comprising one or two Ku heterodimers.

- Authors expose that they were not able to obtain a well fitted model for experimental data obtained by BLI, arguing the low capability of the used model to explain a more complex binding mechanism (lines 485-489). Should the obtained binding constants be taken into account? Or measurements are only qualitative...

-Now lines 528-534: The first BLI experiments were not conclusive, as a result the obtained binding constants are only qualitative in the presented conditions. We added a sentence at the end of the corresponding paragraph to precise this point (lines 537-538).

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7)Authors suggest that the deployed approaches allow them to "characterize the architecture" of the interactions (lines 555-557). However, no structural details or predictions about the studied complexes are provided in this article.

Now line 601: We specified the term architecture by adding "(in term of size and stoichiometry)" in the text as required.

8)In spite of the data provided by the results, a comparative discussion of the obtained KD values is not present in the manuscript. It seems essential in order to evaluate the scope of each of the presented biophysical techniques, as well as consider the use of one or another for each particular case.

We add a comparative discussion of the KD measured by the students and highlight that in the two systems studied the approaches tested allow to measured KD. We compare the difference between the maximal and minimal values obtained for each system (lines 622-630).

9)Some aspects about figures should be noted.

The following figures have been corrected:

- Abbreviations defined in the caption to figure 1 need to be revised. It contains definitions for abbreviations that are not present in the figure (i. e. MT) and it lacks definition for abbreviations that are present (i. e. MM).

-Figure 1: Abbreviations have been updated.

- Graphs in figure 3b should be more understandable. Line grids and marks have be

enlarged.

- Figure 3b: A new figure is proposed with larger line grids and marks.
- Information displayed by figure 4 should be better organised. Sample quantities have to be displayed separated for the measurable parameters or further observations.
- Figure 4: Sample quantities have been displayed separated for the measurable parameters or further observations as suggested.
- Reference to figure 2e in line 398 should be changed by figure 2f.
- Figure 2e/2f references have been corrected.

10)Overall, the use of English should be revised. For instance, some informal expressions have to be changed in order to become more polished or precise. Specially, the repeated use of "amount of material" (lines 436, 569, 579, 589) referring to quantity of sample or expressions like "the same column as above" (line 452) rather than specifying the used material should be avoided.

The use of English has been revised.  
Now lines 481, 591, 633, 642 have been corrected with quantity of sample instead of amount of material.  
Now line 495 has been corrected with the use material.

Reviewer #2: We thank you very much for your comments and we follow all your recommendations.

1)Several of these techniques require a concentration series - the authors have chosen well-characterised systems from their own laboratories, and so the concentration ranges employed to accurately determine binding parameters are already known. However, it might be useful for new users if the authors were to summarise (in a sentence or two) how these concentration ranges were determined. Equally, when discussing AUC the authors mention that  $dn/dc$  and UV extinction coefficients are required. Again, it might be useful for novice users if the authors summarised how these values were determined.

We summarized in a paragraph at the end of section "Sample preparation" how concentration ranges can be determined (lines 356-362).  
AUC required values,  $dn/dc$  and UV extinction coefficients are now described in Materials and Methods section (lines 280-282).

2)I believe that the use of screenshots from analysis software in the figures, whilst often not appropriate, is appropriate in a review of this type. However, the authors should ensure that the resolution of the images is high enough to allow readers to read the axis labels, etc where necessary. Where this isn't possible, legible labels should be added to the figure though other means (illustrator, Inkscape etc).

A higher resolution Figure3b (SECMALS) is proposed with labels that are more legible. In MST figures, legible labels have been added.

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Minor comments have been corrected:

Figure 1 typo - Limited -> Limited.  
Figure 1 typo has been corrected.

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The sentence "The MosBio training school..." has been moved to the following paragraph as suggested (now line 683).

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Now line 692, NMR shift mapping has been added to the text.

Line 644 typo - feedbacks -> feedback

Now line 701 typo has been corrected.

Dear Editors,

We are grateful that you and the reviewers appreciate the study presented in our article and give us the opportunity to send a revised version of our manuscript following the recommendations of the reviewers. You will find below a point-by-point response to the corrections asked by the reviewers. The corresponding changes in the manuscript are coloured in red in the clean version to help visualization. We would like to thank them for their careful analysis of the manuscript and their suggestions that improve the article

**Reviewer #1:** We have incorporated all of your suggestions into our revised manuscript they were very helpful. You will find below the answer to your specific comments:

- 1) *Despite the interest of some of the drawn conclusions—and their indubitable didactical value—, the strongly promotional content of the manuscript should be tempered. For instance, Figure S1 should be removed from the paper—maybe it can be substituted for a link to the website of the course in the main text.*

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[Click here to view linked References](#)

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3 Velazquez-Campoy<sup>5,6,7,8,9</sup>, David Stroebel<sup>10</sup>, Guillaume Bec<sup>11</sup>, Pierre Soule<sup>12</sup>, Christophe  
4 Quétard<sup>13</sup>, Christine Ebel<sup>14</sup>, Alain Roussel<sup>15</sup>, Jean-Baptiste Charbonnier<sup>1</sup>, Paloma Fernández  
5 Varela<sup>1#</sup>

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8 University of Bordeaux, 146 rue Léo Saignat 33076 Bordeaux, France. <sup>3</sup>Bioorganic Chemistry &  
9 Biophysics Core Facility, Max-Planck-Institute of Biochemistry, Martinsried, Germany.  
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18 Moléculaire et Cellulaire (IBMC), Strasbourg, France. <sup>12</sup>NanoTemper Technologies GmbH,  
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22 \*Contributed equally

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24 5078-7102

### 25 **Abstract**

26 Biophysical quantification of protein interactions is central to unveil molecular mechanisms of  
27 cellular processes. Researchers can choose from a wide panel of biophysical methods,  
28 including classical and more novel ones. A real-life proof-of-concept was carried out during an  
29 ARBRE-MOBIEU training school held in June 2019 in Gif-sur-Yvette, France. Twenty European  
30 students benefited from a one-week training with lessons and practical sessions on six  
31 complementary approaches: (i) Analytical UltraCentrifugation with or without a Fluorescence  
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36 protein-protein interaction between an artificial alphaRep binder, and its target protein, also  
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38 (hereafter called Ku), and its cognate DNA ligand. The students acknowledged that the  
39 workshop provided them with a clearer understanding of the advantages and limitations of  
40 the different techniques and will help them in the future to choose the approaches that are  
41 most relevant or informative to their projects.

## 42 **Keywords**

43 Molecular scale biophysics, macromolecular interactions, artificial binders, double-stranded  
44 DNA breaks repair factors

## 45 **Introduction**

46 Macromolecular interactions play a central role in the activation/inactivation of most cellular  
47 mechanisms. These interactions can be measured *in cellulo*, or *in vitro*, and predicted *in silico*.  
48 The classical *in cellulo* methods (such as tap-tag or two-hybrid) allow large-scale studies, but  
49 in order to confirm that a direct interaction occurs between two macromolecules, quantitative  
50 *in vitro* measurements are needed. These measurements allow to characterize interactions  
51 not only in terms of affinity, but also to determine additional kinetic and thermodynamic  
52 parameters, as well as to define the hydrophobic or hydrophilic nature of the interface. They  
53 give access to the stoichiometry of the assembly and allow to map the regions involved by  
54 using different constructs or mutants. *In vitro* measurements can also be useful to evaluate  
55 the role of post-translational modifications or other regulatory events on the formation of  
56 complexes. By *in silico* docking analysis, we can predict the structure of macromolecular  
57 complexes or the impact of functional substitutions helping to optimize experimental design  
58 (Andreani and Guerois, 2014).

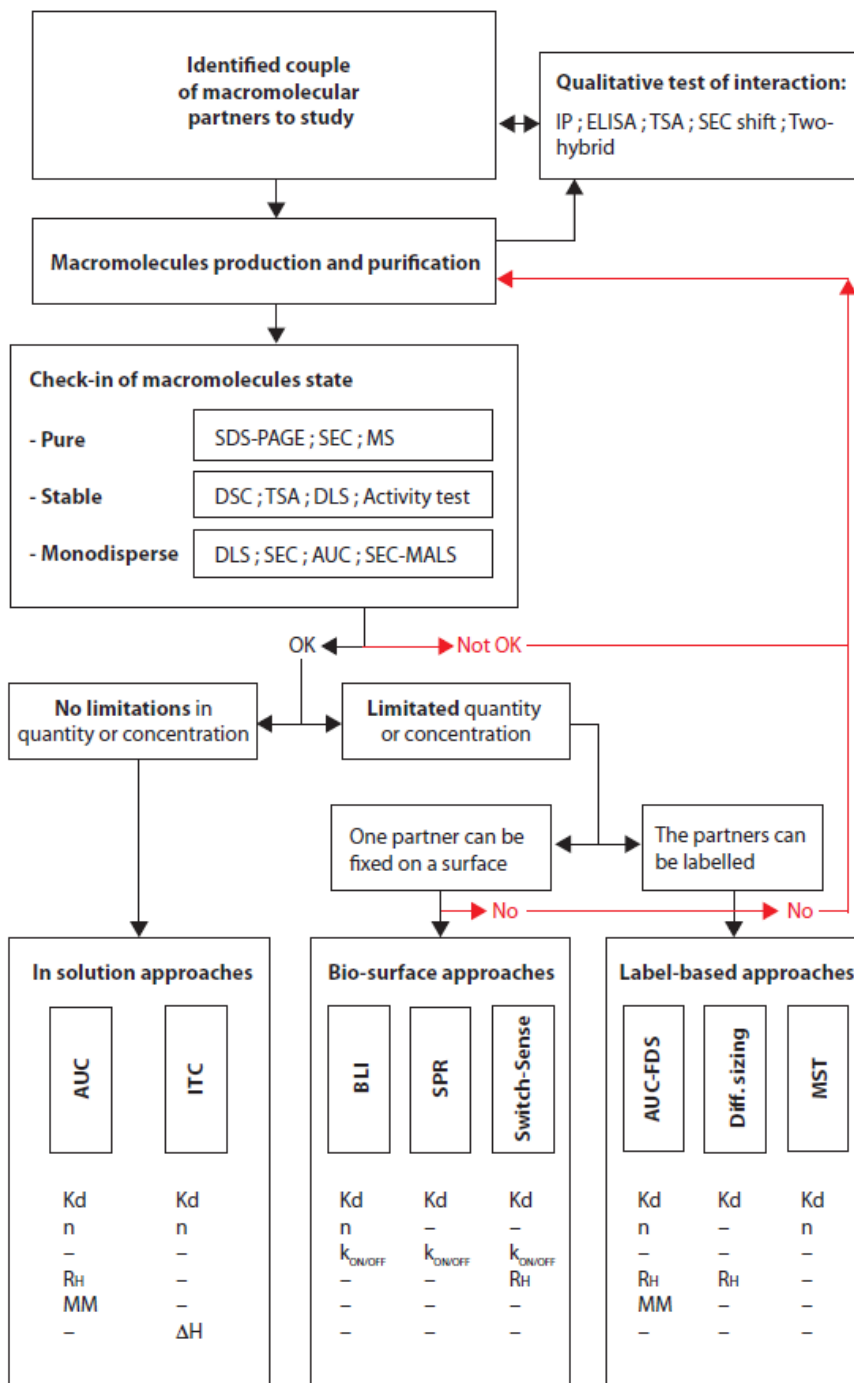
59 The original idea of the project presented here originated during the organization of a  
60 European Training School in Molecular Scale Biophysics (<https://mosbio.sciencesconf.org/>)  
61 within the MOBIEU COST Action. We proposed to the participants to compare different  
62 techniques allowing to measure macromolecular interactions *in vitro*. Nowadays, there is a  
63 large panel of possibilities, and it becomes difficult to choose which technology will be the  
64 best adapted when embarking into a new project. Each approach presents advantages and  
65 drawbacks, and it is therefore difficult for the user to choose from the beginning which one  
66 will be most adapted to the properties of the interaction partners. In the workshop, we

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67 focused on six approaches (Fig. 1). Choosing between the different techniques can be  
68 considered in a progressive manner. If none of the interaction partners can be easily labeled  
69 or immobilized on a surface, approaches in which the macromolecules are in solution (AUC,  
70 ITC, and SEC-MALS) should be favored (Fig. 1, top). However, several of them require large  
71 quantities of biological material. When a partner can be immobilized easily on a bio-surface,  
72 without affecting its function, approaches like BLI, switchSENSE and Surface Plasmon  
73 Resonance (SPR) will be often tested, since they offer the possibility to use small amounts of  
74 the immobilized partner (called ligand) (Fig. 1, middle). Finally, when the partners can be  
75 labeled, again without affecting their function, fluorescent probes can be grafted allowing the  
76 use of reduced amounts of material and facilitated signal analysis (AUC FDS, MST, or  
77 Microfluidic diffusional sizing (MDS)) (Fig. 1, bottom).The choice of the optimal approach may  
78 further rely on additional criteria including the solubility of the partners, the instrument  
79 environment or non-specific interactions with instrument surfaces. Finally, it should be  
80 stressed that it is preferable to perform measurements using several orthogonal techniques  
81 to fully validate and characterize a biological interaction, and specify its features, such as  
82 stoichiometry, kinetics or thermodynamics.

83 **Figure 1. Decisional tree to help choosing the biophysical approach that best suits the study**  
84 **of a specific molecular interaction.**





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86 Six approaches were used in the training school. They can be classified in three main groups: measurements in  
 87 solution and label free (bottom, left), on biosensor with a partner graft on a surface (bottom, middle), and  
 88 methods with a partner labeled with a fluorescent probe (bottom, right). If a quality sample (pure, stable,  
 89 monodisperse) is available in large quantity (up to mg amount) one may start with label-free and in solution  
 90 approaches. Otherwise, if material is limited for one partner, surface approaches are a good alternative. Finally,  
 91 when labeling is possible, MST and AUC-FDS are highly complementary approaches to cross-validate interactions  
 92 measurements. The six approaches give access to different parameters of the interaction and present some  
 93 specific limitations. AUC: Analytical Ultracentrifugation; ITC: Isothermal Titration Calorimetry, SEC-MALS: Size  
 94 Exclusion Chromatography with Multi-Angle Light Scattering; BLI: Bio-Layer Interferometry; MST: MicroScale  
 95 Thermophoresis; AUC-FDS: Analytical Ultracentrifugation with a Fluorescence Detector System; S: Sedimentation  
 96 coefficient; KD, dissociation constant; n, stoichiometry; Dh, hydrodynamic diameter or Rh, hydrodynamic radius;  
 97 kON, kOFF; association and dissociation rates; MT mass transport.

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98 In this training school, we used as examples two different macromolecular interactions  
99 systems, that have been well characterized in our laboratories using several of the approaches  
100 discussed here. One is an interaction between two proteins, and the other a protein-DNA  
101 interaction, both with an affinity in the nanomolar range. Reference data were initially  
102 produced in our laboratories. A group of 20 participants reproduced our measurements during  
103 the five-day MoSBio Training School.

104 The first project comes from P. Minard's team, who uses an original family of artificial  
105 helicoidal repeat proteins, called alphaRep (Guellouz et al., 2013). AlphaRep libraries allow to  
106 select tight binders against a variety of targets by phage display. The alphaRep's are highly  
107 soluble proteins, easily expressed in *E. coli*, which display a very high thermal stability. These  
108 proteins are cysteine-free and, thus do not contain disulfide bonds. They are composed by  
109 repeated motifs made with two antiparallel alpha helices. Clusters of variable side chains,  
110 mainly in the second helix, are positioned on the same face of the motifs. The ensemble of all  
111 these variable motifs forms a library of surfaces from which tight binders can be extracted  
112 against a given target. The alphaRep's have been used for several applications, such as  
113 chaperones for crystallization and structural studies of difficult targets (Valerio-Lepiniec et al.,  
114 2015; Di Meo et al., 2017; Chevrel et al., 2018; Campanacci et al., 2019), as well as in  
115 biophysical and live cell applications (Léger et al., 2019; Prasad et al., 2019; Fernandez et al.,  
116 2020; Léger et al., 2020). New applications of these artificial binders are currently explored in  
117 relation to their ability to be expressed in eukaryotic cells. Here we analyzed the interactions  
118 between two alphaRep's (Rep2 and Rep17) selected against a protein target, (A3), which is  
119 itself an alphaRep. The interaction between the alphaReps has been extensively characterized  
120 before in the laboratory by ITC, SEC-MALS and AUC, but also by Circular Dichroism (CD), SPR  
121 and Fluorescence Resonance energy Transfer (FRET) (not used in this training school)  
122 (Guellouz et al.,2013; Di Meo et al., 2017; Léger et al., 2019). Both A3 / Rep2 and A3 / Rep17  
123 interactions were tested previously to determine which one was the most appropriate for the  
124 training, and the former is weaker than the latter. During the training only A3 / Rep17  
125 interaction was measured.

126 The second project comes from J.B. Charbonnier's team. It concerns proteins involved in DNA  
127 repair, and more precisely, in the classical Non-Homologous End Joining (c-NHEJ), the main  
128 Double-Strand Break (DSB) repair pathway in human. The Ku70/Ku80 (Ku) heterodimer plays

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a central role in the recognition of DSB ends, as it is the first repair factor that interacts with them. Ku binds tightly to DNA ends in a sequence independent manner thanks to its ring-shaped structure (Walker et al., 2001). Ku then iteratively recruits different NHEJ partners (ligase 4, nucleases and polymerases) (Chang et al., 2017; Frit et al., 2019). Ku also contributes to the tethering (synapse) between the two DSB ends to avoid misrepair with other DSB ends. J.B. Charbonnier's team (Tadi et al., 2016; Nemoz et al., 2018) has recently described the recruitment mechanism of some NHEJ factors by Ku at the molecular level. DSBs, despite being deleterious DNA lesions, are generated on purpose during radiotherapy or in genome editing by CRISPR-Cas9. Understanding the molecular basis of the c-NHEJ is thus central to improve these major biotechnological applications. Here we analyzed the interaction between Ku and DNA substrates of different lengths, to determine the most appropriate to the training and to study the threading of Ku on DNA. The characterization of the interaction between Ku and DNA was extensively studied in the laboratory by ITC, MST (Tadi et al., 2016; Nemoz et al., 2018; Gontier, Chapter of MiMB, *in press*), switchSENSE, AUC, BLI (data not published) and, more recently by other techniques not used during the training school (SPR, MDS or FRET). One Ku occupies about 18 bp on DNA. Several Ku molecules can thread on DNA when the size of the DNA is longer than 18bp, and a long DNA can be covered by one Ku molecule every 18bp.

We present in this article the results obtained by 20 students during a European Training School in Molecular Scale Biophysics that took place from June 3<sup>rd</sup> to 7<sup>th</sup>, 2019 at I2BC at Gif-sur-Yvette, France. We report the protocols used to analyze the two systems under study (protein/protein and protein/DNA interactions) using the six biophysical methods mentioned above. The results obtained by the students during this week are shown and compared to our previous published data when available. Finally, we compare the advantages and drawbacks of the different approaches used during this training and present some feedbacks from the students, allowing to have a global overview of the pros and cons of these six complementary biophysical approaches.

156 **Materials and Methods**

157 **Biophysical approaches in solution that do not require labeling of an interaction partner**

158 **Size Exclusion Chromatography with Multi-Angle Laser Light Scattering detection (SEC-**  
159 **MALS):** SEC-MALS allows to determine the absolute molar mass of the components of a  
160 protein/multiprotein sample. It indicates if elution peaks are homogenous in term of protein  
161 composition or if they are composed of mixtures, either of different oligomers or of different  
162 conformers. SEC-MALS allows size determination if an online Dynamic Light Scattering (DLS)  
163 detector is included in the setup (Folta-Stogniew, 2006). We used an HPLC system from  
164 *Shimadzu* coupled to a MALS detector (miniDAWN TREOS) plus a DLS detector (QELS), and a  
165 refractometer (optilab T-rEX) from *Wyatt technologies* (Fig. S1). To run an experiment, one  
166 needs 1 L of running buffer to equilibrate the column and detectors, and 2 mg/mL of protein  
167 (for MW of 20 kDa), down to 0.5 mg/mL if the MW is higher than 150 kDa. Depending of the  
168 column used, the volume of the sample (30  $\mu$ L for Bio-SEC-3 *Agilent*, 50  $\mu$ L for KW-803/804  
169 *Shodex*, and 100  $\mu$ L for Superose 6, Superdex 75 or Superdex 200 increase 10/300 GL *Cytiva*)  
170 and the time of elution can differ. A run (including an equilibration step and a control BSA  
171 sample) takes typically a few hours and consumes about 40-200  $\mu$ g of sample.

172 **Isothermal Titration Calorimetry (ITC):** ITC allows the direct, and thorough thermodynamic  
173 characterization of interactions between molecules in solution with no limitation of partner  
174 size (Holdgate, 2001; Krell, 2008; Velazquez-Campoy and Freire, 2006) and without labeling.  
175 ITC is an equilibrium solution technique to quantify dissociation constants ( $K_D$ ) values, but also  
176 other interaction parameters (enthalpy, entropy, stoichiometry, and heat capacity). ITC is not  
177 affected by the optical properties of the samples, but may be very sensitive to the composition  
178 of the buffer (e.g. presence of DMSO or mismatches between solutions). For the ITC  
179 experiments, we used three instrument models: VP-ITC, ITC200 and PEAQ-ITC all from  
180 *Malvern Panalytical* (Fig. S1). One of the interacting partner is placed in a cell (1.4 mL for the  
181 VP-ITC, 200  $\mu$ L for the ITC200 and PEAQ-ITC) and the other in a syringe (300  $\mu$ L for the VP-ITC,  
182 40  $\mu$ L for the ITC200 and PEAQ-ITC). Sequential injections are made from a syringe (5-10  $\mu$ L  
183 for the VP-ITC, 1-2  $\mu$ L for the ITC200 and PEAQ-ITC). In the data described here, we used 80-  
184 990  $\mu$ g of the molecule in the cell, and 130-320  $\mu$ g of the molecule in the syringe. The transient  
185 heat effect due to complex formation (and other potential unspecific phenomena) upon  
186 partner injection is measured as the titration progresses, from which the binding isotherm is

187 constructed.

## 188 **Biophysical approaches with a partner immobilized on a surface**

189 **Bio-Layer Interferometry (BLI):** BLI is a fast, high throughput and label-free technology for  
190 measuring biomolecular interactions analyzing the interference pattern of white light  
191 reflected from a layer of immobilized macromolecules on a biosensor tip and an internal  
192 reference (Abdiche et al., 2018). It enables real-time analysis for determination of affinity,  
193 kinetics and concentration, with one of the binding partners immobilized onto the biosensor  
194 surface (ligand) and the other in solution (analyte). This microfluidic-free technology is  
195 particularly adapted for performing binding assays in crude lysates or cell culture media. We  
196 used an Octet RED96e during the training, and previously a RED384, both from *FortéBio* (Fig.  
197 S1). We used 1-5 µg/mL of ligand to load NTA sensors for 20-120 s for low density or 120-600  
198 s for high density, and a range of at least 7 (2-fold dilutions starting at 100-200 nM) of the  
199 analyte with an association time of 600-900 s. We consumed per run about 8 µg of protein A3,  
200 and 400 ng of 42 bp biotinylated DNA for the immobilization, and about 2-14 µg of respective  
201 analytes. The concentration of ligand could be reduced (at least by 2) in favor of a longer  
202 incubation. The consumption of the analyte, depends on the affinity of the interaction, as the  
203 concentrations used should range from  $K_D / 20$  to  $10 \times K_D$ .

204 **switchSENSE:** switchSENSE technology is based on short DNA nanolevers (48 bp in our case),  
205 which are immobilized on gold electrodes in a microfluidic channel. The intrinsically negatively  
206 charged DNA nanolevers can be electrically actuated (“switched”) on the gold surface to  
207 oscillate at high frequencies (Knezevic et al., 2012). A switchSENSE microfluidic biochip  
208 contains four flow channels, each containing six gold electrodes. Switching of the DNA is  
209 mediated by alternating the voltage across the gold surface. The motion of the levers is  
210 tracked in real time (µs scale) via time-resolved single photon counting detecting a fluorescent  
211 probe present on the immobilized DNA strands. The complementary DNA strands can be  
212 cross-linked to a ligand via amine or thiol coupling or click-chemistry. By hybridization of this  
213 conjugated complementary strand to the surface-tethered DNA nanolever, the surface is  
214 functionalized with the ligand of interest. Upon binding of an analyte, the hydrodynamic  
215 friction of the levers is affected and subsequently the movement of the levers is slowed down.  
216 This change in switching speed is used by the system to determine the size ( $D_h$ ) or  
217 conformational changes of ligands and complexes. The kinetics of molecular interactions ( $k_{ON}$ ,

218  $k_{OFF}$ ,  $K_D$ ) can be followed using two measurement modes: dynamic or static. In the first case,  
219 analyte binding is measured through the change of the oscillation rate of the electrically  
220 actuated DNA nanolevers (changes in dynamic response). In the second case, the DNA  
221 nanolevers are kept at an upright position, in a constant electric field, and analyte in close  
222 proximity to a dye can alter the local chemical environment resulting in a fluorescence change  
223 (also called Fluorescence Proximity Sensing). Binding is then measured thanks to the  
224 fluorescence intensity variation of the functionalized nanolever. For the switchSENSE  
225 experiments, we used a DRX<sup>2</sup> device from *Dynamic Biosensors* with two LED light sources (for  
226 the excitation of red and green fluorophores) (Fig. S1). In all cases, a hundred  $\mu\text{g}$  of protein  
227 were enough to generate cross-linked complementary strands (cNL-DNA) for several round  
228 of experiments, since one measurement needs only 40  $\mu\text{L}$  of 100 nM DNA-protein conjugate.  
229 A sizing measurement classically takes less than an hour. The amount of analyte needed for a  
230 kinetic experiment depends on the overall affinity, which delimits flow rates, and  
231 association/dissociation times to be used. During association, a too slow flow rate can be the  
232 cause for mass transport limitation effect and during dissociation an inadequate flow rate can  
233 result in re-binding effects. Here we used flow rates of 100-500  $\mu\text{L}/\text{min}$ , association times of  
234 80-300 s, and 1500 s of dissociation time. In sample quantities, we used 150 ng of the cross-  
235 linked ligands, and 2-20  $\mu\text{g}$  of its partner for a series of 3 concentrations.

### 236 **Biophysical approaches with a labeled partner**

237 **Analytical UltraCentrifugation with Fluorescence Detection (AUC-FDS):** AUC is a powerful  
238 technique for the characterization of macromolecules and macromolecular self- and hetero-  
239 association processes in solution. It was used here with labeled protein or DNA, but it can be  
240 used with non-labeled material like SEC-MALS and ITC. An analytical ultracentrifuge is a high-  
241 speed centrifuge equipped with one or more detectors (absorbance/interference,  
242 fluorescence) allowing to monitor sedimentation in real time. Two types of complementary  
243 experiments can be performed, sedimentation velocity and sedimentation equilibrium (Zhao  
244 et al., 2013). Sedimentation velocity experiments allow to determine the size distribution of  
245 species, their aggregation and oligomerization, sedimentation coefficients, hydrodynamic  
246 radius, shape and molar masses, their stoichiometry and  $K_D$  (by isotherm fitting sedimentation  
247 coefficients measured at various concentrations). Sedimentation equilibrium is suited for well-  
248 defined samples, and gives molar mass and  $K_D$  information. The centrifugation speed can be

249 set between 600 and 260 000 g allowing to study all sizes of macromolecules. The duration of  
1 sedimentation experiments ranges from 2 h to several days (Fig. S1). For the AUC-FDS  
2 250  
3 experiments we used a ProteomeLab XL-I ultracentrifuge from *Beckman Coulter* equipped  
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5 with a Fluorescence Detection System (FDS) from *AVIV Instruments* (Fig. S1). A sedimentation  
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7 velocity experiment requires 100-450  $\mu\text{L}$  of sample at 0.3 to 1.5 OD, for  
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9 absorbance/interference detection, and 5-60 nM concentration, for fluorescence detection,  
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11 whereas sedimentation equilibrium requires 130  $\mu\text{L}$  at 0.2 to 0.5 OD. In interference, the limit  
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13 of detection is 0.1 mg/mL, but in absorbance it depends on sample extinction coefficients. To  
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15 produce the data described here, we used about 1-3.5  $\mu\text{g}$  of the fluorescent molecule and 8-  
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17 80  $\mu\text{g}$  of the non-fluorescent molecule.  
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20 259 **Microscale Thermophoresis (MST):** MST is a novel technology for the analysis of biomolecules  
21 based on the modification of fluorescence intensity induced by the temperature, due to  
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23 Temperature Related Intensity Changes (TRIC) and the directed movement of particles in a  
24 261  
25 microscopic temperature gradient (thermophoresis) (Asmari et al., 2018; Jerabek-Willemsen  
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27 et al., 2011). Thermophoresis is influenced by a combination of changes at the level of the  
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29 hydration shell, shape, charge..., (all the parameters that influence the Soret coefficient) of  
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31 biomolecules, which result in differences of movement along the temperature gradient as well  
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33 as the brightness of the fluorescent tag. MST provides information on the binding affinities  
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35 with good accuracy and sensitivity in the pM to mM range. This technology allows  
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37 immobilization-free measurement of interactions in any buffer and complex biological liquid,  
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39 but requires one of the two partners to be labeled with a fluorescent dye to measure protein-  
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41 protein interactions (there is also a label free instrument that detects the intrinsic  
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43 fluorescence of proteins). Any size of unlabeled molecules can be used (from ions, to large  
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45 proteins). We used a Monolith NT.115 blue/green and red/blue from *NanoTemper*  
46 272  
47 *Technologies* (Fig. S1). MST experiments, performed in capillaries, require a low sample  
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49 consumption (200  $\mu\text{L}$  at 20 nM of the fluorescent molecule, and 20  $\mu\text{L}$  of the non-fluorescent  
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51 molecule at the highest concentration needed, depending on the expected  $K_D$ ). To produce  
52 275  
53 the data described here, we used about 50-350 ng of the fluorescent molecule and 6-12  $\mu\text{g}$  of  
54 276  
55 the non-fluorescent molecule.  
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## 57 278 **Sample preparation**

59 279 AlphaReps are recombinant proteins produced by standard overexpression procedures in  
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280 *E.coli* (Guellouz et al., 2013). For our experiments, we used the following alphaRep proteins:  
281 A3, which is the target and Rep17 and Rep2, which are the binders. Because A3 forms  
282 homodimers at high concentration, it was used as ligand for real-time biosensors approaches  
283 (BLI, switchSENSE) and was the labeled partner in AUC-FDS and MST experiments. In the  
284 conditions used, we assumed A3 is a monomer, as the simultaneous presence of the  
285 monomeric and dimeric forms would make the analysis difficult.

286 Ku is a recombinant protein produced by standard overexpression procedures in insect cells  
287 (Nemoz et al., 2018). For our experiments, we used different lengths of dsDNA depending on  
288 the assay format. Shorter DNA are not adapted for biosensor approaches, since a DNA too  
289 close to the surfaces will hinder its interaction with Ku. DNA is practical to work with, because  
290 it is easy to modify and is commercially available. For immobilized on BLI biosensors and for  
291 detection in AUC-FDS and MST, we ordered biotinylated and 5-Carbofluorescein (5-FAM)-  
292 labeled DNA oligonucleotides, respectively.

293 To perform biophysical characterization measurements, samples of high purity, stability and  
294 monodispersity are needed (Fig. S2). All proteins were dialyzed to eliminate glycerol from the  
295 storage buffer, which can interfere with the measurements. We chose the pH and ionic  
296 strength of the buffer for the best solubility of the samples. To simplify as much as possible,  
297 we used the dialysis buffer (20 mM Tris-HCl pH 8, 150 mM NaCl, 5 mM  $\beta$ -mercaptoethanol) as  
298 running buffer in most experiments. Due to the particularities of each approach and  
299 measurement devices, there are some limitation in the buffer choice. In some cases, blocking  
300 agents were required, such as 1mg/mL BSA, especially for low protein concentrations (in the  
301 nM range), to prevent surface adsorption, as well as detergents, such as 0.1 % Tween-20, to  
302 reduce surface tension (capillaries, chip, biosensors). For AUC-FDS, we had to avoid Tris-HCl  
303 or HEPES above 20 mM, which can cause problems at 230 nm wavelength, and be aware of  
304 other absorbent molecules (nucleotides, old DTT or  $\beta$ -mercaptoethanol). For MST experiment,  
305 we used the commercial MST buffers (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 10 mM  $MgCl_2$ ,  
306 0.05 % Tween-20) for Ku-DNA and, Roti<sup>®</sup>-Stock 1x PBS (10 mM  $Na_2HPO_4$ , 2 mM  $KH_2PO_4$  pH  
307 7.6137 mM NaCl, 2.7 mM KCl, 0.005% Tween 20) for A3-Rep17. For switchSENSE sizing  
308 experiments  $D_h$  estimation, a low salt buffer is required (10 mM Tris-HCl, 40 mM NaCl, 0.05 %  
309 Tween-20, 50  $\mu$ M EGTA, 50  $\mu$ M EDTA).

## 310 **Results**

### 311 **Biophysical measurements of alphaRep interactions**

312 We characterized the interaction of A3 with its binders Rep2 and Rep17 using the following  
313 techniques presented in the Fig. 1: in solution approaches, which do not require labeling (SEC-  
314 MALS and ITC), in solution approaches that require labeled protein or DNA (MST and AUC-  
315 FDS), and surface approaches (BLI and switchSENSE). The students performed the  
316 measurements presented here during the training (Fig. 2, Fig. S3 and Table S1). The studied  
317 interactions data represent limited value due to students measuring and protein  
318 concentration as common denominators, but added benefits of putting the techniques in  
319 context. For most of them, additional measurements and controls are needed. When  
320 available, we mention the values that have been reported for some approaches in previous  
321 articles.

### 322 **AlphaRep interactions measured by label free in solution approaches**

323 Due to time limitations, the SEC-MALS experiments were performed in our platform prior to  
324 the training. We used a Superdex 200 increase 10/300 GL column (*Cytiva*), and compared  
325 three different runs with A3 alone, Rep2 alone and A3-Rep2 complex at 2 mg/mL  
326 concentration each. A3 eluted as a dimer with a molar mass of  $44.8 \pm 0.4$  kDa and, a  
327 hydrodynamic radius of  $3.0 \pm 0.2$  nm. Rep2 eluted as a monomer with a molar mass of  $11.6 \pm$   
328  $0.9$  kDa. The Rep2 concentration was insufficient, in view of the small molar mass, to make  
329 accurate sizing measurement by DLS. Finally, the A3-Rep2 complex eluted before the free  
330 proteins, with a molar mass of  $65.5 \pm 0.4$  kDa and hydrodynamic radius of  $4.0 \pm 0.2$  nm (Fig.  
331 2b). The stoichiometry was determined by using the protein conjugate method (Loiseau et al.,  
332 2017). Accurate mass can be calculated from MALS data with this method, which uses two  
333 concentration detectors simultaneously (a refractometer and UV spectrophotometer), and  
334 information about refractive index increment ( $dn/dc$ ) and UV extinction coefficient of each  
335 component. In our case, the complex corresponds to one dimer of A3 and two Rep2  
336 molecules.

337 Preparatory ITC experiments were performed in our platform on a PEAQ-ITC instrument with  
338 A3 in the cell at  $18 \mu\text{M}$  and Rep17 in the syringe at  $187 \mu\text{M}$  (Fig. S3a). Three different groups  
339 of students did a triplicate measurement (Fig. 2c) during the training using the same

340 instrument. We observed bi-phasic thermograms, and isotherms were fitted using a model of  
341 two identical binding sites with cooperativity. This cooperativity could stem from the  
342 propensity of A3 to form homodimers at  $\mu\text{M}$  concentration as shown by SEC-MALS (Freire et  
343 al., 2009; Vega et al., 2015). We could estimate two  $K_D$  values, the first  $K_D$  corresponds to an  
344 interaction with a tight affinity ( $K_{D1}$  0.5 - 0.7 nM) and the second one to a weaker affinity ( $K_{D2}$   
345 16 – 32 nM). With the obtained parameters for each site, described in the table at the bottom  
346 of thermograms, an average global affinity (Wyman and Gill, 1990) could be calculated  
347 (geometric mean of both dissociation constants:  $K_{D,av} = \sqrt{K_{D1} \times K_{D2}}$ ), which for each assay  
348 was 3.9 nM, 3.1 nM, and 3.2 nM, close to that obtained with other techniques. Data were  
349 analyzed during the training using the Origin software and the new PEAQ ITC software for  
350 comparison.

### 351 **AlphaRep interactions measured by biophysical approaches with a partner immobilized on** 352 **a surface**

353 Preliminary BLI experiments were performed before the training on an Octet RED384  
354 instrument where His-tagged A3 protein was captured on Ni-NTA sensors at 5  $\mu\text{g}/\text{mL}$  for 20 s  
355 and Rep17 concentration ranges from 200 nM to 1.56 nM. In these conditions, in which A3 is  
356 most probably in a monomeric form, the calculated kinetic rates were  $k_{ON}$  of  $1.3 \cdot 10^{+5} \text{ M}^{-1}\text{s}^{-1}$ ,  
357  $k_{OFF}$  of  $5.9 \cdot 10^{-3} \text{ s}^{-1}$  and  $K_D$  of 22.4 nM (data not shown). We observed that the fit was not correct  
358 with a single site model and that residuals showed systematic errors. During the training, a  
359 duplicate experiment was performed on an Octet RED96e in a buffer containing 1 mg/mL BSA  
360 and 0.1 % Tween-20 to limit the non-specific binding. In this case, the fitting was improved  
361 (Fig. 2d). We obtained the following preliminary values:  $k_{ON}$  of  $2.6 \pm 0.7 \cdot 10^{+5} \text{ M}^{-1}\text{s}^{-1}$ ,  $k_{OFF}$  of  $7.6$   
362  $\pm 0.7 \cdot 10^{-4} \text{ s}^{-1}$ ,  $K_D$  of  $3.2 \pm 1.1 \text{ nM}$ . The  $K_D$  measured from the plateau of the curve (steady state)  
363 was of  $3.3 \pm 0.8 \text{ nM}$ , close to the one obtained from kinetics (Fig. S3c).

364 For the switchSENSE experiments, we first coupled A3 with the DNA strand (cNL-B48), which  
365 was complementary to the surface-tethered DNA strand on the chip. In this case, the surface-  
366 tethered DNA strand was labeled with a red fluorescent probe (NLB48-red dye). The DNA-  
367 protein conjugate was purified using an anion-exchange-chromatography (Fig. S3d, top left).  
368 We then hybridized the A3-cNLB48 with the NLB48 on the sensor surface at a concentration  
369 of 100 nM. The hybridization step could be monitored in real-time by measuring the

1 370 fluorescence increase (Fig. S3d, top right). Preliminary kinetic experiments were done using  
2  
3 371 the dynamic mode (switching of the nanolevers) in our platform. Rep2 alphaRep was used as  
4  
5 372 analyte at three different concentrations ranging from 100 nM to 11.1 nM. Preliminary kinetic  
6  
7 373 rates were calculated from a global fit for the three concentrations:  $k_{ON}$  of  $2.40 \pm 0.05 \cdot 10^{+6} M^{-1} s^{-1}$ ,  
8  
9 374  $k_{OFF}$  of  $6.86 \pm 0.23 \cdot 10^{-4} s^{-1}$  and  $K_D$  of  $0.29 \pm 0.01$  nM (Fig. S3d, bottom, left). For each analyte  
10  
11 375 concentration, it was possible to perform a sizing experiment to measure the hydrodynamic  
12  
13 376 diameter of the ligand A3, before or after association with Rep2. The  $D_h$  of the dimeric form  
14  
15 377 of A3 calculated from the crystal structure (PDB 6FT) is found to be equal to 4.8 nm. Due to  
16  
17 378 protein dilution, we expect A3 to be present as a monomer on the DNA conjugated strand.  
18  
19 379 Indeed, the  $D_h$  of  $3.0 \pm 0.1$  nm measured in our switchSENSE experiments is compatible with  
20  
21 380 a monomeric form of A3 (Fig. S3d, bottom, right). The  $D_h = 3.8 \pm 0.1$  nm measured for the  
22  
23 381 complex A3-Rep2 corresponds to an increase of 0.8 nm when compared to the hydrodynamic  
24  
25 382 diameter of the monomeric form of A3 (Fig. S3d, bottom, left). During the training, kinetic  
26  
27 383 experiments in static mode (nanolevers in up position) were performed, in the same  
28  
29 384 conditions as before for Rep2, but with Rep17 alphaRep as ligand. The calculated kinetic values  
30  
31 385 measured in this case were in the same range than the ones obtained previously:  $k_{ON}$  of  $2.35$   
32  
33 386  $\pm 0.17 \cdot 10^{+6} M^{-1} s^{-1}$ ,  $k_{OFF}$  of  $2.30 \pm 0.34 \cdot 10^{-3} s^{-1}$  and  $K_D$  of  $0.98 \pm 0.16$  nM (Fig. 2e). During the  
34  
35 387 training, students did not have time to run a sizing experiment.

36  
37 388 Comparable analysis using SPR classical technology, were carried out in our platform using a  
38  
39 389 ProteON XPR36 instrument from *Biorad* (data not published, Fig. S3b).

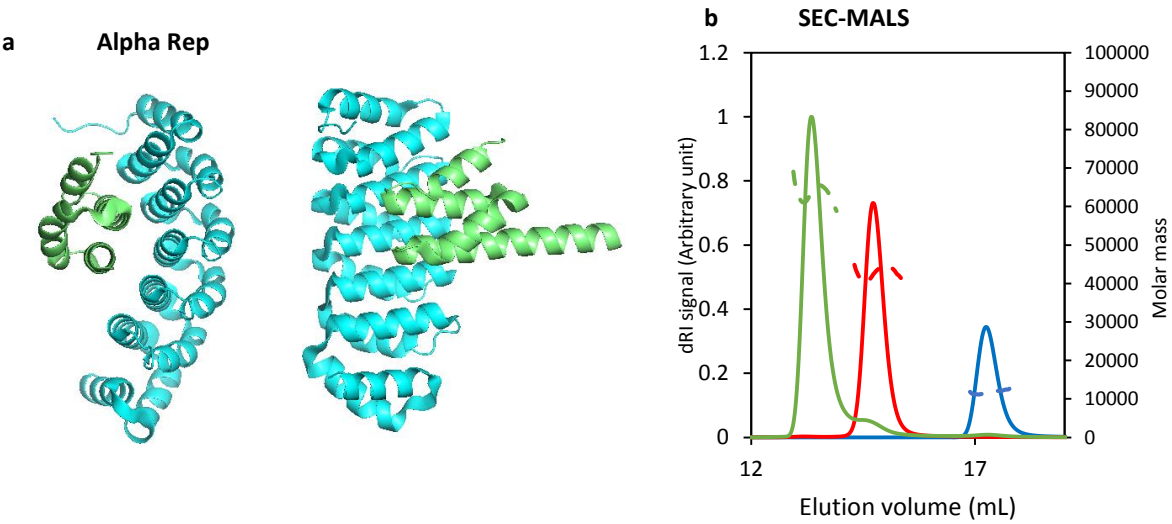
### 42 390 **AlphaRep interactions measured by biophysical approaches with a labeled partner**

43  
44 391 Because AUC-FDS experiment were time consuming, data were collected before the training  
45  
46 392 in our platform. We first labeled A3 with the dye NT495 using the commercial *NanoTemper*  
47  
48 393 Monolith Protein Labeling Kit BLUE-NHS (Amine Reactive). In these conditions, we were able  
49  
50 394 to use a low concentration of A3 (5 nM). At this nanomolar concentration, A3 is a monomer  
51  
52 395 as observed before by switchSENSE. We used variable concentrations of Rep17 from 0.5 nM  
53  
54 396 to 0.5  $\mu M$ , and an An-50 Ti rotor at 42 000 rpm (130 000 g) speed. The sedimentation  
55  
56 397 coefficients for A3 and A3-Rep17 complex were  $2.16 \pm 0.08$  S and  $2.96 \pm 0.05$  S, respectively  
57  
58 398 and the calculated  $K_D$  at equilibrium was  $13.4 \pm 1.8$  nM (Fig. 2e).

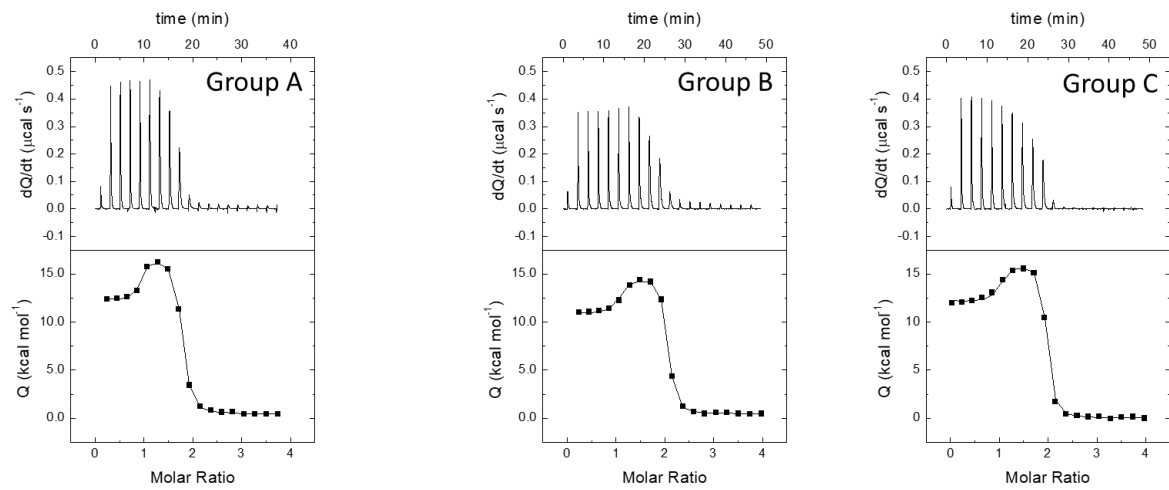
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399 Finally, for the MST experiment, we decided to use another dye from *NanoTemper* Monolith  
400 His-Tag Labeling Kit Red-tris-NTA 2<sup>nd</sup> Generation, this site-specific non covalent labeling  
401 substantially improved the signal and respected better the integrity of protein. In this MST  
402 experiment, labeled A3 was at 70 nM, and the highest concentration of Rep17 was 8  $\mu$ M. The  
403 titration curves we obtained allowed us to measure a  $K_D$  of  $8.0 \pm 4.3$  nM (Fig. 2g). Using the  
404 labeled A3 produced for the AUC-FDS at 20 nM did not provide us with substantial results due  
405 to high inhomogeneity in the sample preparation (Fig S3e), thus leading to a strong noise and  
406 suggesting that this labeling of A3 influences the interaction on the time scale of the MST  
407 measurement. Interestingly, we observed a titration curve with a  $K_D$  at  $10.5 \pm 2.4$   $\mu$ M, which  
408 could be linked to an additional interaction between A3-Rep17 complexes at the highest  
409 Rep17 concentration of 288  $\mu$ M or some steric hindrance of the fluorescent probe. Orthogonal  
410 confirmation would help to better understand the origin of this  $K_D$  difference, unfortunately  
411 none of the other approaches were performed at such high concentration.

412 **Figure 2. Biophysical measurements of alphaRep interactions.**



**c ITC**

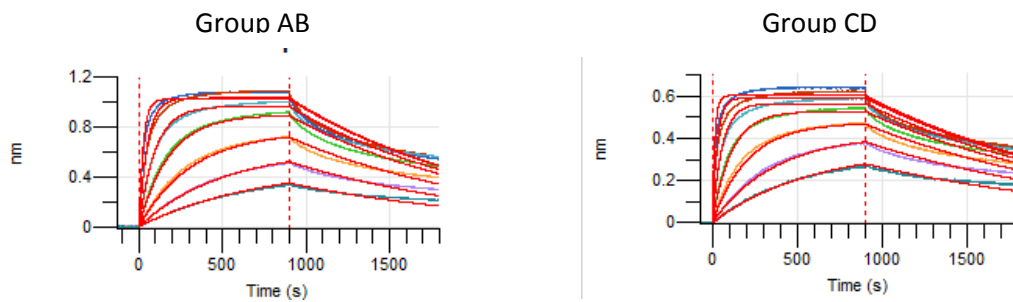


	K (M <sup>-1</sup> )	K <sub>D</sub> (nM)	ΔH (kcal/mol)	α	Δh (kcal/mol)	n
Group A	2.1 · 10 <sup>9</sup>	0.48	12.0	0.015	4.3	0.84
Group B	1.7 · 10 <sup>9</sup>	0.59	10.5	0.036	3.6	0.98
Group C	1.5 · 10 <sup>9</sup>	0.66	12.2	0.041	3.8	0.94

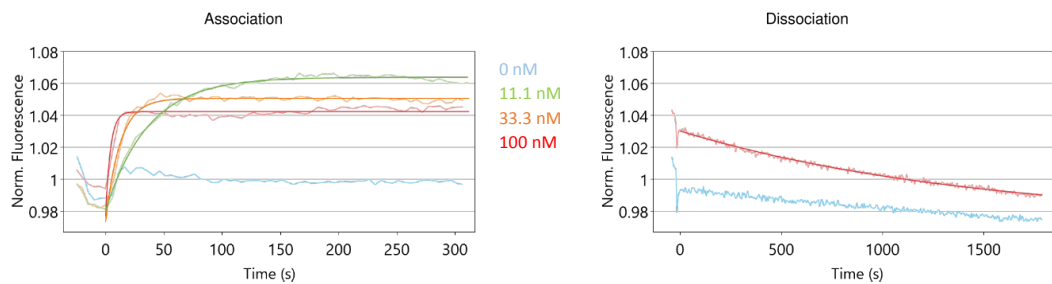
  

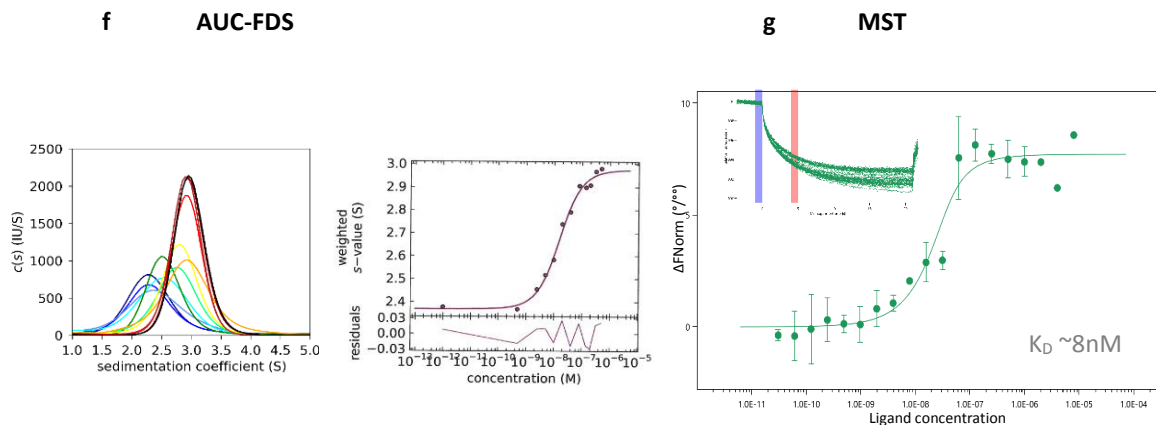
	K <sub>D1</sub> (nM)	ΔH <sub>1</sub> (kcal/mol)	K <sub>D2</sub> (nM)	ΔH <sub>2</sub> (kcal/mol)
Group A	0.48	12.0	32	16.3
Group B	0.59	10.5	16	14.1
Group C	0.66	12.2	16	16.0

**d BLI**



**e switchSENSE**





14 413 a) Crystal structure of A3 alphaRep (a-A3, blue) in complex with Rep2 (bA3-2, green) (PDB: 4JW2) at 90° view  
 15 414 (left). b) SEC-MALS analysis. Elution profiles and molar masses of Rep2 in blue, A3 in red, and the complex of A3-  
 16 415 dimer and two molecules of Rep2 in green. c) PEAQ-ITC data of A3-Rep17 interaction obtained during the training  
 17 416 for three groups (A, B and C).  $K$ ,  $K_D$ , and  $\Delta H$  were the association constant, the dissociation constant, and the  
 18 417 interaction enthalpy of either of the two binding sites when the A3 dimer was unoccupied.  $\alpha$  and  $\Delta h$  were the  
 19 418 cooperative interaction constant and the cooperative interaction enthalpy, which reflect the binding cooperative  
 20 419 phenomenon (a factor that modulates the binding affinity and a term that modulates the binding enthalpy to the  
 21 420 second site when there was a site already occupied, respectively).  $n$  was the active (or binding-competent)  
 22 421 fraction of protein, since the stoichiometry was already included in the model.  $K_{D1}$  and  $\Delta H_1$  were the intrinsic  
 23 422 site-specific dissociation constant and binding enthalpy for the first binding site ( $K_{D1} = K_D$ ,  $\Delta H_1 = \Delta H$ ).  $K_{D2}$  and  $\Delta H_2$   
 24 423 were the intrinsic site-specific dissociation constant and binding enthalpy for the second binding site ( $K_{D2} = K_{D1} /$   
 25 424  $\alpha$ ,  $\Delta H_2 = \Delta H_1 + \Delta h$ ). d) Octet RED96e BLI kinetic analysis of A3-Rep17 interaction in duplicate. Colors code of  
 26 425 Rep17 concentration: 3.13 nM (teal), 6.25 nM (purple), 12.5 nM (orange), 25 nM (green), 50 nM (cyan), 100 nM  
 27 426 (red), 200 nM (blue). e) switchSENSE kinetics analysis of A3-Rep17 interaction on dynamic mode, at three Rep17  
 28 427 concentrations. Normalized association (left) and dissociation (right) data were represented in function of time.  
 29 428 f) AUC-FDS distribution of sedimentation coefficients (left) with labeled A3 at 5 nM and increasing concentrations  
 30 429 of Rep17 from 0.5 nM to 0.5  $\mu$ M, obtained with the program SEDFIT (Schuck, 2000) and GUSI (Brautigam, 2015).  
 31 430 Colors code of Rep17 concentrations: 0  $\mu$ M (navy blue), 0.5 nM (blue), 1 nM (cornflower blue), 2 nM (cyan), 4  
 32 431 nM (green), 8 nM (springgreen), 16 nM (yellow), 32 nM (orange), 64 nM (red), 128 nM (maroon), 256 nM (brown)  
 33 432 and 0.5  $\mu$ M (black). AUC-FDS  $K_D$  calculation (right), by fitting with isotherm tool in the program SEDPHAT. g) MST  
 34 433 analysis with red fluorophore. Inset, MST traces, MST on used was 10 s (red band).

38 434 In summary, the students obtained preliminary results in one week with the six approaches  
 39 435 that were consistent in stoichiometry, and to a lesser extent, in kinetic rates or affinity (Table  
 40 436 S1). They could observe that the required amount of material, ligand or analyte, for each  
 41 437 approach differs significantly. They also observed that some approaches gave highly  
 42 438 complementary information on the system studied (Fig. S3). For example, ITC shows that the  
 43 439 interaction is cooperative when A3 alphaRep is used at high concentration needed for ITC  
 44 440 (18 $\mu$ M). This cooperativity, linked to the dimeric state of A3, was not observed with  
 45 441 monomeric ligand immobilized on BLI or switchSENSE. They observed higher  $K_D$  values with  
 46 442 AUC-FDS and MST, where one of the partner is labeled, reflecting possible steric hindrance  
 47 443 between the fluorophore and the interaction site. This was particularly true with the covalent  
 48 444 amine labeling strategy where the  $K_D$  measured by MST was three order of magnitude higher.



## 445 **Biophysical measurements of Ku-DNA interactions**

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3 446 We determined the interaction of the heterodimeric full-length protein Ku with four double-  
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5 447 strand DNAs (dsDNAs) of different lengths: 18 bp, 42 bp, 48 bp, and 200 bp (Fig. 3, Fig. S4). Ku  
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7 448 binds DNA through its ring-shaped structure (Fig. 3a). The main observations made during the  
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9 449 training for the Ku-DNA interactions, using the same six approaches, as for the alphaRep  
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11 450 interactions, are shown in Fig. 3 and Table S2.

## 12 13 451 **Ku-DNA interactions measured by label-free in solution biophysical approaches**

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15 452 For SEC-MALS, we used the same column as above and ran first a quadruplicate experiment  
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17 453 with Ku alone, resulting in an average molar mass of 144.4 kDa (Fig. S4a). We then ran a  
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19 454 duplicate experiment with Ku and a 1.2 excess of 42 bp DNA. We obtained three peaks: the  
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21 455 first corresponded to 2 Ku : 1 DNA complex (average Mw of 306.2 kDa), the second  
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23 456 corresponded to 1 Ku : 1 DNA (average Mw of 171.0 kDa), and the last corresponded to the  
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25 457 excess of dsDNA alone (average Mw of 26.1 kDa). This suggests that there is an equilibrium  
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27 458 between 2:1 and 1:1 Ku-DNA complexes (Fig. 3b) with stoichiometries determined by using  
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29 459 the protein conjugated method (Loiseau et al.; 2017).

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31 460 For ITC experiments, we used in our laboratory two different sizes of dsDNA, 18 bp and 42 bp  
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33 461 (Gontier, Chapter of MiMB, *in press*). Due to time limitations students repeated the  
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35 462 measurement only with the 42 bp. We used a VP-ITC instrument which consumes more  
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37 463 sample, but was more sensitive to study these protein-DNA interactions. The heat effects were  
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39 464 positive (endothermic interaction) and small ( $0.2 \mu\text{cal}\cdot\text{sec}^{-1}$ ) (Fig. 3c). Students did two runs,  
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41 465 the first at 20  $\mu\text{M}$  and the second at 40  $\mu\text{M}$  concentration of DNA in the syringe, and in both  
42  
43 466 cases Ku at 4  $\mu\text{M}$  in the cell. We obtained a mean  $K_D$  at equilibrium of  $3.7 \pm 0.7 \text{ nM}$  with a  
44  
45 467 molar ratio of  $0.34 \pm 0.02$  and a  $\Delta H$  of  $24.4 \pm 2.7 \text{ kcal/mol}$ . The molar ratio was in good  
46  
47 468 agreement with a ratio of 0.5 expected for the interaction of two Ku molecules with a DNA of  
48  
49 469 42 bp. No evidence for cooperativity was observed. All the data were analyzed using the Origin  
50  
51 470 software and the new PEAQ ITC software for comparison.

## 52 53 471 **Ku-DNA interactions measured by biophysical approaches with a partner immobilized on a** 54 55 472 **surface**

56  
57 473 Preparatory BLI experiments were performed on an Octet RED384 instrument. Firstly, we tried  
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59 474 the same strategy that was used for the A3 alphaRep protein, i.e. captured Ku heterodimer on  
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1 475 Ni-NTA sensors. We did not observe any binding with an 18 bp DNA as analyte (data not  
2 476 shown). We hypothesized that the DNA binding site may not be accessible when Ku is  
3  
4 477 immobilized through its His-tag. We therefore tested another strategy, which relied on the  
5  
6 478 capture of a biotinylated 42 bp DNA on Streptavidin sensors (at 10 nM for 120 s), which were  
7  
8 479 then incubated with Ku protein as analyte (at concentrations in 200 nM to 1.56 nM range). In  
9  
10 480 these conditions, we observed an interaction, with an estimated  $K_D$  around 40 nM (data not  
11  
12 481 shown). We repeated the experiment in duplicate during the training on an Octet RED96e  
13  
14 482 using an optimized buffer with 1mg/mL BSA and 0.1 % Tween-20, and Ku protein  
15  
16 483 concentrations ranging from 200 nM to 3 nM. The results of the two runs were consistent,  
17  
18 484 with apparent kinetic rates,  $k_{ON}$  of  $1.91 \pm 0.02 \cdot 10^{+6} \text{ M}^{-1}\text{s}^{-1}$ ,  $k_{OFF}$  of  $7.17 \pm 0.33 \cdot 10^{-4} \text{ s}^{-1}$ , and a  $K_D$   
19  
20 485 of  $0.37 \pm 0.01 \text{ nM}$  (Fig. 3d). However, the deviation between the fitted curves and the  
21  
22 486 experimental data was high, indicating that the interaction mechanism was more complex  
23  
24 487 than a simple 1:1 binding. For instance, the association and the dissociation processes could  
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26 488 be limited by the diffusion of Ku towards and from the biosensor surface (mass transport  
27  
28 489 limitation). We therefore analyzed the concentration-dependence of the steady state  
29  
30 490 responses and measured a  $K_D$  of  $5.2 \pm 0.2 \text{ nM}$  (Fig. S4b). As several curves did not reached a  
31  
32 491 steady state, this  $K_D$  value could however be overestimated and experiments should be  
33  
34 492 reproduced with longer association times.

35  
36 493 Preliminary switchSENSE experiments were done before the training by forming an 80 bp  
37  
38 494 dsDNA on the sensor. For that, we hybridized an 80-mer DNA with a 32-mer DNA. This DNA  
39  
40 495 was complementary to the NL48 ssDNA on the chip (Fig. S4c, top, left). We performed kinetic  
41  
42 496 analyses in duplicate using static mode and Ku protein at 500 nM concentration. We obtained  
43  
44 497 the following kinetic rates:  $k_{ON}$  of  $9.3 \pm 0.1 \cdot 10^{+4} \text{ M}^{-1}\text{s}^{-1}$ ,  $k_{OFF}$  of  $2.8 \pm 0.1 \cdot 10^{-4} \text{ s}^{-1}$ , and  $K_D$  of  $3.1 \pm$   
45  
46 498  $0.1 \text{ nM}$  (Fig. S4c, bottom). We monitored the dissociation over a long time (5000 s) to measure  
47  
48 499 a significant proportion of dissociated Ku molecules. In between, we performed a sizing  
49  
50 500 measurement. The calculated value ( $D_h = 8.9 \pm 0.3 \text{ nm}$ ) was in good agreement with the one  
51  
52 501 obtained from the crystal structure of the Ku-DNA complex (PDB: 1JEQ;  $D_h = 8.9 \text{ nm}$ ) (Fig. S4c,  
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54 502 top, right). During the training, we performed experiments in dynamic mode using a 48 bp  
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56 503 DNA (without overhang). We used Ku concentration range from 200 nM to 22.2 nM with 1/3  
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58 504 serial dilution this time. In these conditions, the protein was closer to the fluorophore at the  
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60 505 tip of the 48 bp DNA on the chip. We measured in these conditions a  $k_{ON}$  of  $2.6 \pm 0.2 \cdot 10^{+6} \text{ M}^{-1}$

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6 506  $s^{-1}$ , a  $k_{OFF}$  of  $2.3 \pm 0.3 \cdot 10^{-3} s^{-1}$ , and a  $K_D$  of  $1.0 \pm 0.2$  nM (Fig. 3e). During the training, students  
7  
8 507 did not have time to run a sizing experiment.  
9

10  
11 508 Comparable preliminary data were obtained by SPR in our platform using a ProteON XPR36  
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13 509 instrument from *Biorad* (Fig. S4d, left), and in Institut Pasteur platform using a Biacore T200  
14  
15 510 instrument from *Cytiva* (Fig. S4d, right).  
16

### 17 18 511 **Ku-DNA interactions measured by biophysical approaches with a labeled partner**

19  
20 512 For AUC-FDS, we used an 18 bp DNA labeled with fluorescein (FAM) in 5'. We titrated Ku  
21  
22 513 protein from 0.5 nM to 0.5  $\mu$ M concentrations with 60nM of DNA. The sedimentation profile  
23  
24 514 showed two species, corresponding to the DNA alone ( $2.15 \pm 0.05$  S) and to the 1:1 protein-  
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26 515 DNA complex ( $7.25 \pm 0.15$  S), and we were able to determine the  $K_D$  at equilibrium ( $12.9 \pm 3.2$   
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28 516 nM) (Fig. 3f).  
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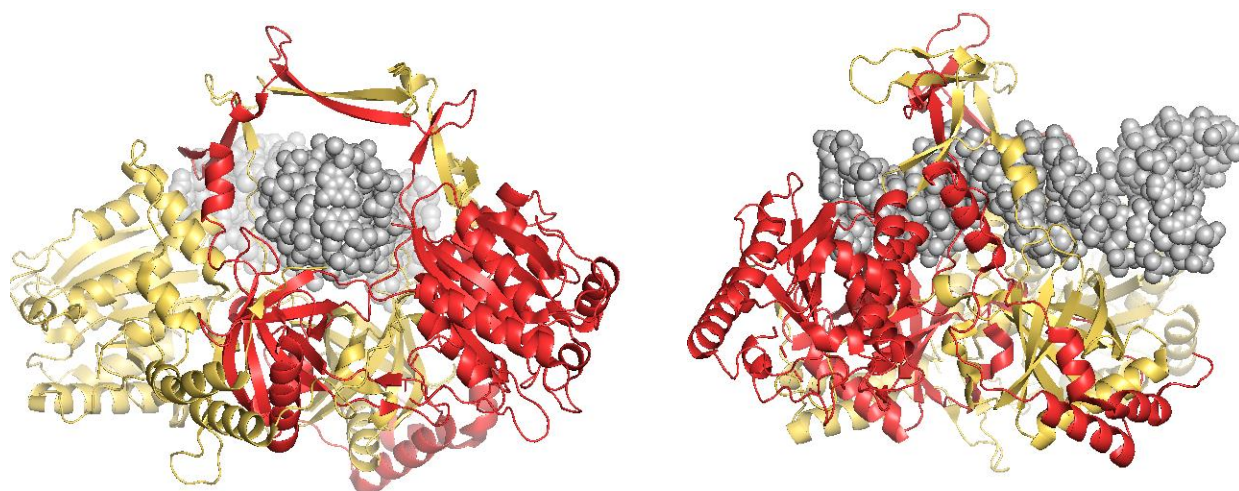
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31 517 For MST, we used the same labeled 18 bp DNA as for AUC-FDS, but at 10 nM concentration;  
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33 518 and titrated Ku from 2  $\mu$ M concentration. Training participants performed four runs in total,  
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35 519 in duplicates or triplicate measurements (Fig. 3g). We could see some variability in the curves  
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37 520 that might originate from the pipetting of the different students. All curves were fitted globally  
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39 521 resulting in a  $K_D$  of 2.8 nM.  
40

41 522 Comparable data were obtained with a novel technology, microfluidic diffusional sizing (MDS)  
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43 523 in collaboration with Fluidic Analytics (Fig. S4e). The Fluidity One-W instrument measures the  
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45 524 rate of diffusion of macromolecules under steady state laminar flow in a microfluidic chip. In  
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47 525 a diffusion chamber with two parallel streams, the migration of a labeled partner depends on  
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49 526 its size. At the end, the streams are re-split and from the ratio of the fluorescence between  
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51 527 them,  $R_h$  is calculated. Then, changes in average size after titration of an unlabeled binding  
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53 528 partner against the labeled partner, give a binding curve to calculate a  $K_D$  value.  
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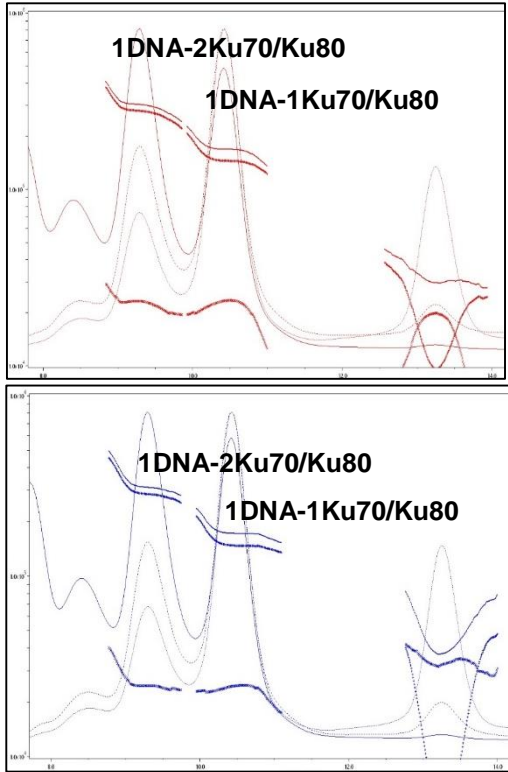
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529 **Figure 3. Biophysical measurements of Ku-DNA interactions.**

a **Ku-DNA**

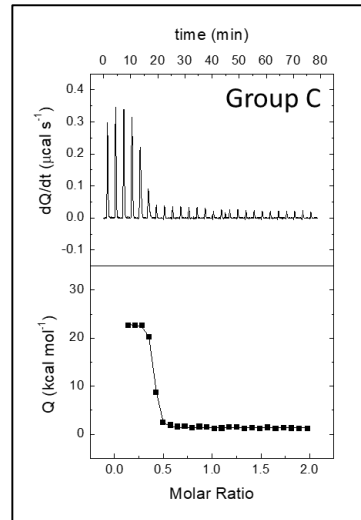
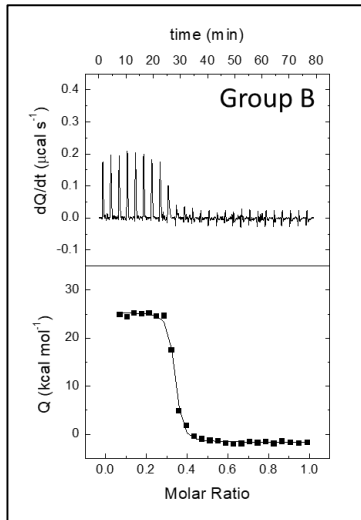


**b SEC-MALS**



		Peak 1	Peak 2	Peak 3
Group C	Protein molar mass	279.1	145.7	9.6
	DNA molar mass	23.3	23.3	19.9
	Total molar mass	302.3	169.0	29.6
Group D	Protein molar mass	285.3	148.4	4.8
	DNA molar mass	24.7	24.6	32.3
	Total molar mass	310.1	172.9	37.1

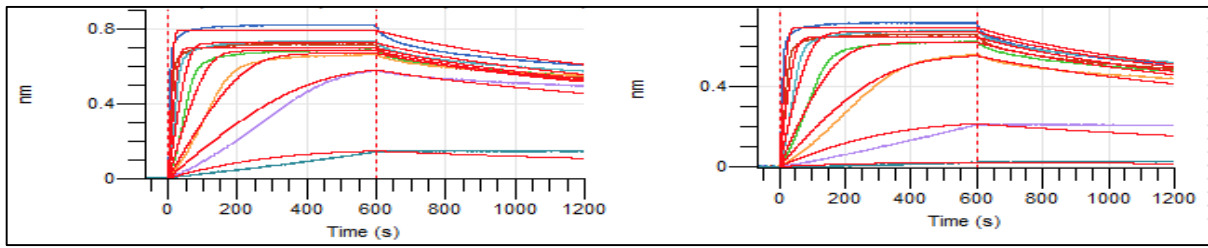
**c ITC**



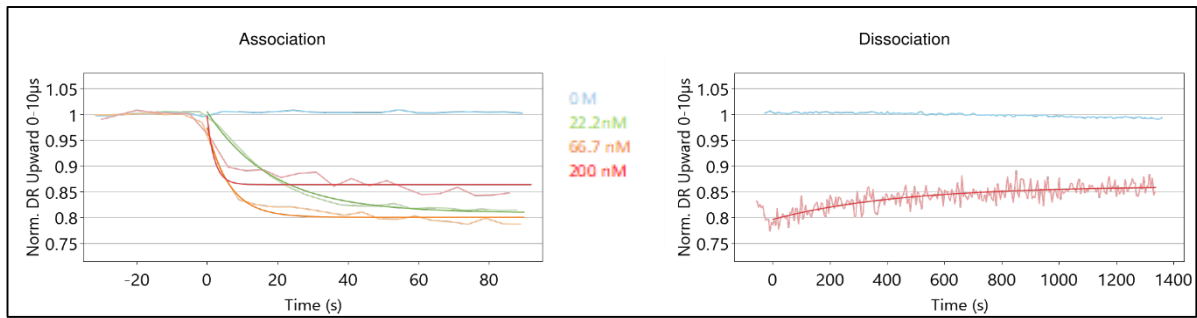
	K (M <sup>-1</sup> )	K <sub>D</sub> (nM)	ΔH (kcal/mol)	n
Group B	3.4 · 10 <sup>8</sup>	3.0	27.1	0.32
Group C	2.3 · 10 <sup>9</sup>	4.3	21.7	0.37

**d BLI: Group AB and Group CD**

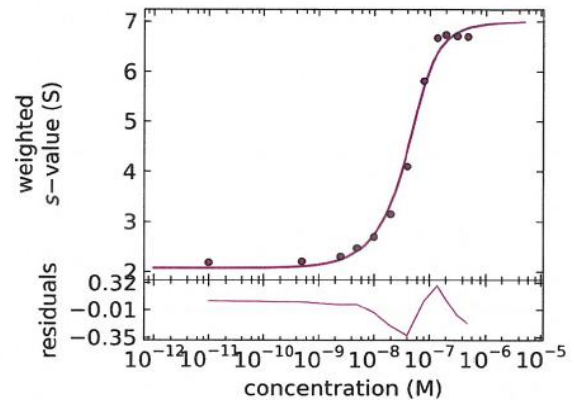
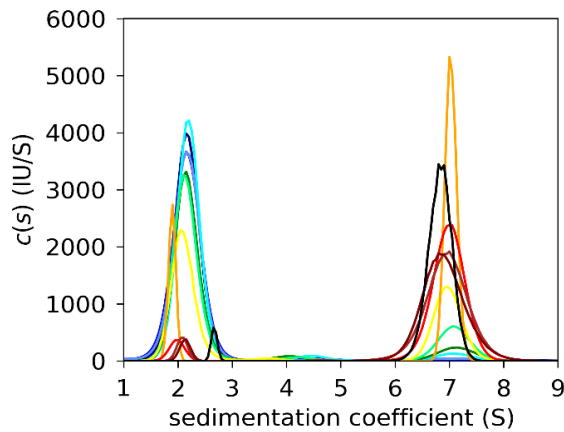
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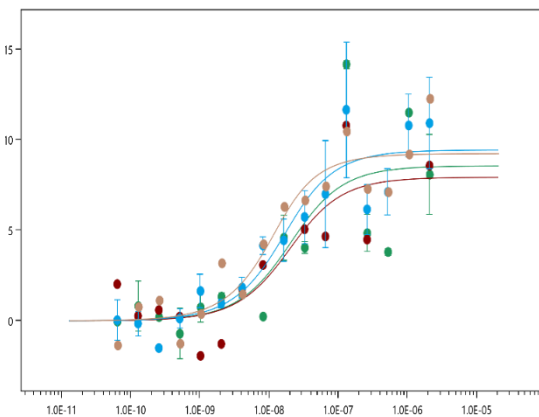
**e** switchSENSE



**f** AUC-FDS



**g** MST



GROUPS	Excitation Power	$K_D$	$K_D$ Confidence	Std. Error of Regression	Signal to Noise
A1-B1	60	1.53 $10^{-8}$	1.86 $10^{-8}$	2.77	3.33
A2	40	1.49 $10^{-8}$	1.73 $10^{-8}$	2.01	4.33
B2-C2-D2	100	1.14 $10^{-8}$	7.26 $10^{-9}$	1.61	6.31
D1	100	6.15 $10^{-9}$	4.3 $10^{-9}$	1.56	6.37

a) Crystal structure of the Ku- DNA complex (PDB: 1JEQ). View down the DNA helix (left) and side view (right), with Ku70 colored in red, Ku80 in yellow cartoons and DNA in grey spheres. b) SEC-MALS elution profiles and

532 molar masses of Ku-DNA complex in duplicate. Representation of elution volumes in mL and molar mass in g/mol.  
533 c) VP-ITC data in duplicate. d) Octet RED96e BLI kinetics data in duplicate. Colors code of Ku concentration: 3.13  
534 nM (teal), 6.25 nM (purple), 12.5 nM (orange), 25 nM (green), 50 nM (cyan), 100 nM (red), 200 nM (blue). e)  
535 switchSENSE dynamic mode data for three Ku concentrations. F) AUC-FDS measurement of Ku-DNA interaction.  
536 AUC-FDS distribution of sedimentation coefficients (left) with labeled DNA at 60 nM and increasing  
537 concentrations of Ku from 0.5 nM to 0.5  $\mu$ M, obtained with the program SEDFIT (Schuck, 2000) and GUSI  
538 (Brautigam, 2015). Colors code of Ku concentration: 0  $\mu$ M (navy blue), 0.5 nM (blue), 1 nM (cornflower blue), 2  
539 nM (cyan), 4 nM (green), 8 nM (springgreen), 16 nM (yellow), 32 nM (orange), 64 nM (red), 128 nM (maroon),  
540 256 nM (brown) and 0.5  $\mu$ M (black). AUC-FDS  $K_D$  calculation by fitting with isotherm tool in the program SEDPHAT  
541 (right). g) MST analysis. Representation of ligand concentration against  $\Delta F_{\text{Norm}} \text{ } ^\circ/\text{ } ^\circ$  (left). Superposition of  
542 several run in the similar conditions, but different excitation power and students. Summary of MST data (right).

543 In summary, the students obtained also for the Ku-DNA project results consistent in  
544 stoichiometry, and in the same range for kinetic rates or affinity (Fig. 3, Table S2). As for the  
545 alphaRep project, they observed that the amount of material varies between the different  
546 approaches. ITC, SEC-MALS and AUC-FDS were very efficient for measuring the stoichiometry  
547 of Ku on DNA with one Ku molecule bound every 18-21bp DNA. They observed that  
548 measurements on surfaces, either by BLI or switchSENSE, were successful only when the DNA  
549 was immobilized. They could observe that switchSENSE allows a quite accurate estimation of  
550 the Ku size (Fig. S4d, bottom). Oligonucleotides with a fluorescent probe in 5' or 3' are  
551 inexpensive and allow to follow the interaction between a fluorescent DNA and a protein quite  
552 easily by MST or AUC-FDS, without major steric hindrance between the fluorescent probe and  
553 the interaction sites.

## 554 Discussion

555 The study presented here compares six different *in vitro* biophysical approaches to  
556 characterize the architecture and binding parameters of two different types of complexes  
557 (protein-protein and protein-DNA). It was designed to provide a rather complete overview of  
558 six different techniques in a short period. During a week, 20 participants performed this study  
559 in the context of a MoSBio Training School (ARBRE-MOBIEU COST Action). During the first day  
560 of the training school, experts of each field presented the projects, the theory and examples  
561 of applications for the three classical techniques (AUC, SEC-MALS, ITC), and the three more  
562 recent ones (MST, BLI, switchSENSE). An additional presentation about, sample quality  
563 control, a relevant subject regarding reproducibility of experimental measurements,  
564 completed the first day training (Raynal et al., 2014). The remainder of the week was  
565 dedicated fulltime to practical sessions where the participants could perform experiments on  
566 instruments, analyze results and discuss with experts. All six approaches, except AUC, and SEC-  
567 MALS for the alphaRep interaction, were successfully used during the training school to study

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568 two important types of macromolecular interactions (protein-protein and protein-DNA). The  
569 training allowed the students, first, to compare the quantity of material consumed for each  
570 technique, and, second, to understand the parameters that can be measured by each of them  
571 (Fig. 4). This first edition of the MoSBio Training School was positively assessed by both experts  
572 and participants. It was a unique opportunity to compare advantages and limitations of this  
573 large ensemble of techniques. A new edition will be scheduled soon. The participants were  
574 able to use all instruments quite easily by themselves, with the exception of AUC, which  
575 requires a little more expertise and longer run times. Finally, this study showed that a  
576 nanomolar range affinity is easy to be assessed with all tested techniques. Nevertheless, this  
577 is not the case when we want to measure lower  $K_D$  (pM) or higher  $K_D$  ( $\mu$ M) values.

578 The results presented here for the alphaRep-proteins and Ku-DNA complexes highlight the  
579 advantages and drawbacks of each approach. The amount of required material was not limited  
580 in our case, but the participants clearly observed that the amounts of protein and DNA used  
581 for each approach are very different (Fig. 4). AUC, SEC-MALS and ITC are the most sample-  
582 consuming techniques. ITC200 and PEAQ-ITC were an important progress for ITC in this regard,  
583 but we observed that for interactions with very weak heat exchange like Ku-DNA, the more  
584 sensitive, but more sample-consuming VP-ITC was still needed to obtain good results. Surface  
585 methods like BLI and switchSENSE require only small amounts of the immobilized protein. For  
586 the study of high affinity systems (in the nM range) like those characterized here, the  
587 consumption of analyte is small too. For weaker affinities (higher  $K_D$  in the  $\mu$ M range) the  
588 amount of analyte will however rise significantly to cover concentrations from 1/10 of the  $K_D$   
589 to 10 times the  $K_D$ . The MST technique consumes very small volumes of material once the  
590 labeling step is successfully achieved. Anyway, the time and sample consumption for a given  
591 technique must be evaluated not just for a single successful experiment, but also considering  
592 the experimental design and optimization stage.



593 **Figure 4. Comparison of results obtained for the alphaReps and the Ku-DNA interaction with**  
 594 **the six approaches.**

SEC- MALS	<b>AlphaReps</b> <ul style="list-style-type: none"> <li>✓ 200 µg A3, 200 µg Rep2</li> <li>➤ Molar mass</li> <li>➤ Stoichiometry: (A3-dimer): 2 Rep2</li> </ul>	ITC	<b>AlphaReps</b> <ul style="list-style-type: none"> <li>✓ 80 µg A3, 130 µg Rep17</li> <li>✓ Biphasic curve</li> <li>➤ Cooperativity (A3 dimer)</li> <li>➤ <math>K_{D1}</math>, <math>K_{D2}</math>, <math>\Delta H</math>, <math>-\Delta S</math></li> </ul>
	<b>Ku-DNA</b> <ul style="list-style-type: none"> <li>✓ 40µg of DNA, 200 µg Ku</li> <li>➤ Molar mass</li> <li>➤ Stoichiometry: 1 Ku /18-21 bp</li> </ul>		<b>Ku-DNA</b> <ul style="list-style-type: none"> <li>✓ 990 µg Ku, 320 µg DNA</li> <li>✓ Biphasic curve</li> <li>➤ Stoichiometry: 1 Ku /18-21bp</li> <li>➤ <math>K_D</math>, <math>\Delta H</math>, <math>-\Delta S</math></li> </ul>
BLI	<b>AlphaReps</b> <ul style="list-style-type: none"> <li>✓ 8 µg A3, 2 µg Rep17</li> <li>✓ A3-monomer</li> <li>➤ <math>K_D</math>, <math>k_{ON}</math>, <math>k_{OFF}</math></li> </ul>	switchSENSE	<b>AlphaReps</b> <ul style="list-style-type: none"> <li>✓ 150 ng A3, 2-20 µg Rep17</li> <li>✓ A3-monomer</li> <li>✓ Size (<math>D_h</math>)</li> <li>✓ <math>K_D</math>, <math>k_{ON}</math>, <math>k_{OFF}</math></li> </ul>
	<b>Ku-DNA</b> <ul style="list-style-type: none"> <li>✓ 400 ng DNA, 14 µg Ku</li> <li>✓ Failed with Ku immobilized</li> <li>➤ <math>K_D</math>, <math>k_{ON}</math>, <math>k_{OFF}</math></li> </ul>		<b>Ku-DNA</b> <ul style="list-style-type: none"> <li>✓ 150 ng DNA, 2-20 µg Ku</li> <li>✓ Ligand is the nanolever</li> <li>✓ Size (<math>D_h</math>)</li> <li>➤ <math>K_D</math>, <math>k_{ON}</math>, <math>k_{OFF}</math></li> </ul>
AUC-FDS	<b>AlphaReps</b> <ul style="list-style-type: none"> <li>✓ 1 µg A3, 8 µg Rep17</li> <li>➤ Sedimentation coefficient</li> <li>➤ Stoichiometry</li> <li>➤ <math>K_D</math></li> </ul>	MST	<b>AlphaReps</b> <ul style="list-style-type: none"> <li>✓ 620 ng A3, 6 µg Rep17</li> <li>✓ One labeling failed</li> <li>➤ <math>K_D</math></li> </ul>
	<b>Ku-DNA</b> <ul style="list-style-type: none"> <li>✓ 3.5 µg DNA, 80 µg Ku</li> <li>✓ DNA easily labeled</li> <li>➤ Sedimentation coefficient</li> <li>➤ Stoichiometry</li> <li>➤ <math>K_D</math></li> </ul>		<b>Ku-DNA</b> <ul style="list-style-type: none"> <li>✓ 50 µg DNA, 12 µg Ku</li> <li>✓ DNA easily labeled</li> <li>➤ <math>K_D</math></li> </ul>

595 The experiments performed by the students on the two systems of study with the six approaches were  
 596 successful. Quantity of materials used for the different approaches are very different. Here are the amount  
 597 needed per run. The training highlighted the parameters that can be deduced from the different approaches.

598 All experimental approaches used in this study can provide  $K_D$  values of the interaction.

599 Interestingly, they provide additional information that may orient the users to one or the  
 600 other according to the main questions raised in their specific project. In the two examples we  
 601 studied, the stoichiometry issue is an important point, since the A3 alphaRep is a dimer when  
 602 its concentration is in the  $\mu M$  range, while it is a monomer in the nM range. According to this  
 603 oligomeric state, we can observe some cooperative binding of its partner or not, or some  
 604 influence on the binding parameters. In this regards, ITC, SEC-MALS and AUC allow  
 605 characterizing the ratio between the dimeric alphaRep target and its binders or between Ku  
 606 and DNA of increasing lengths, and confirm the information provided by their crystal

607 structures. The sizing measurement in switchSENSE can also provide some information  
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2 608 regarding the stoichiometry/conformation.

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4 609 Kinetic parameters are important data to estimate the half-time of a complex, which is an  
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6 610 interaction parameter gaining attention recently. BLI and switchSENSE (as well as SPR) proved  
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8 611 to be efficient in this study to determine  $k_{ON}$  and  $k_{OFF}$ . We observed that the switchSENSE  
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10 612 allows to characterize very tight interactions with small  $k_{OFF}$  values ( $k_{OFF}$  about  $10^{-4}$  s) by  
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12 613 monitoring the dissociation over a long time period (5000 s). Thermodynamic parameters  
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14 614 (enthalpy, entropy and Gibbs free energy) also provide key information about macromolecular  
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16 615 interactions, notably about the polar/apolar nature of the interface between a protein and its  
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18 616 ligand. The Ku-DNA thermograms for example indicate that the interaction is entropically  
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20 617 driven with a positive and unfavorable enthalpy probably linked to desolvation of the DNA  
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22 618 and Ku surface upon binding. The MoSBio training school over a week with 20 students proved  
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24 619 to be a very rich occasion for such discussions.

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26 620 The choice of one technique over another depends mainly on the questions one wants to  
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28 621 answer. Some practical issues can also guide the choice of the method, such as the quantity  
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30 622 and solubility of protein and ligands available, the possibility to immobilize or to label one of  
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32 623 the interaction partners, and the time available. The presence of an instrument and experts  
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34 624 in or nearby the user is another criterion. Noteworthy, all these approaches are available in  
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36 625 many research institutions in Europe, especially in those engage in Structural Biology studies.  
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38 626 If needed, these approaches are also available through network infrastructures, like Instruct  
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40 627 in Europe or FRISBI in France. Finally, there are research networks, like ARBRE-MOBIEU,  
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42 628 allowing and fostering fruitful exchanges with experts in the different fields.

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44 629 Studying macromolecular interactions *in vitro* are extremely complementary to *in cellulo*  
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46 630 analyses. Interactions observed in cells or in cellular extracts need often need *in vitro*  
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48 631 validation and target engagement to confirm the specificity of the proposed interaction.  
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50 632 Training school as the one described in this article are central to further disseminate the  
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52 633 importance of biophysical studies in cellular biology laboratories. This training school  
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54 634 contribute to initiate students from various fields on biophysical approaches and show in a  
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56 635 short period the added-value of quantitative measurements of protein interactions.

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58 636 By mutagenesis or HDX-MS, one can determine the residues involved in the interaction, and  
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60 637 structural analysis can yield information about their topology. One can play with the  
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638 experimental conditions (pH, ionic strength, temperature) to expand the knowledge about the  
639 nature of the interaction under study (electrostatic and/or hydrophobic, exo- or endothermic,  
640 presence/absence of conformational changes, coupling with additional binding equilibria).  
641 Furthermore, pharmacological studies require this kind of biophysical approaches. Finally,  
642 molecular scale biophysics data can be also used to implement *in silico* simulations to predict  
643 other interactions in basic research. A second edition of this Training School will be held as  
644 soon as possible. The extremely positive feedbacks from the students, and from the academic  
645 experts and industrials participating in this school highlights the need for a better  
646 understanding of the theoretical bases and hands-on experimental practical of such a panel  
647 of biophysical methods.

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**745 Ethics approval** Not applicable.

**746 Consent to participate** The authors consent to participate to this project.

**747 Consent for publication** The authors consent to publish the work reported in this paper.

**748 Availability of data and material** Data can be obtained by requesting the corresponding  
**749 author.**

**750 Code availability** Not applicable.

**751 Authors' contributions** PFV, PE, SU, CE, AVC, AR, JBC authors contributed to the study  
**752 conception and design.** Material preparation, data collection and analysis were performed by  
**753 CV, MAN, SU, PE, AVC, DS, GB, PS, CQ, CE, AR.** The first draft of the manuscript was written by  
**754 PFV** and all authors commented on previous versions of the manuscript. All authors read and  
**755 approved the final manuscript.**

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**766 possible,** and finally the keynote speakers, Julie Ménétreay and Terence Strick who shared their  
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**768 (<https://www.pluginlabs-universiteparisclay.fr/fr/results/keywords/PIM>),** while some  
**769 others** were performed in Institut Pasteur, PFBMI platform.

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4 770 **Supplementary material**

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6 771 **Figures**

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8 772 **Figure S1. Flyer of the Molecular Scale Biophysics (MoSBio):**

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Gif-sur-Yvette, France  
June 3<sup>rd</sup> -7<sup>th</sup>, 2019

ARBRE-MOBIEU Training School on  
**Macromolecular Interactions**  
comparing *in vitro* methodologies

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COST

**AUC**  
Analytical UltraCentrifugation

**ITC**  
Isothermal Titration Calorimetry

**SEC/MALS**  
Size Exclusion Chromatography  
Multi-Angle Light Scattering

**From classical to innovative approaches:  
Principles and Practice**

**switchSENSE**  
Molecular Dynamics Measurement

**MST**  
MicroScale Thermophoresis

**BLI**  
Bio-Layer Interferometry

**I2BC organisers:** CHARBONNIER Jean-Baptiste, FERNANDEZ-VARELA Paloma,  
MENETREY Julie, MINARD Philippe, NICAISE-AUMONT Magali & VELOURS Christophe

**External Speakers and Trainers:**  
BARON Bruno, BRULE S bastien, ENGLAND Patrick & RAYNAL Bertrand, (Institut Pasteur, Paris, France)  
BEC Guillaume & ENNIFAR Eric (IBMC, Strasbourg, France)  
EBEL Christine (IBS, Grenoble, France)  
ROUSSEL Alain (AFMB, Marseille, France)  
STRICK Terence & STROEBEL David (ENS, Paris, France)  
UEBEL Stephan (MPI Biochemistry, Martinsried, Germany)  
VELAZQUEZ CAMPOY Adrian (BIFI, Zaragoza, Spain)

<https://mosbio.sciencesconf.org/>

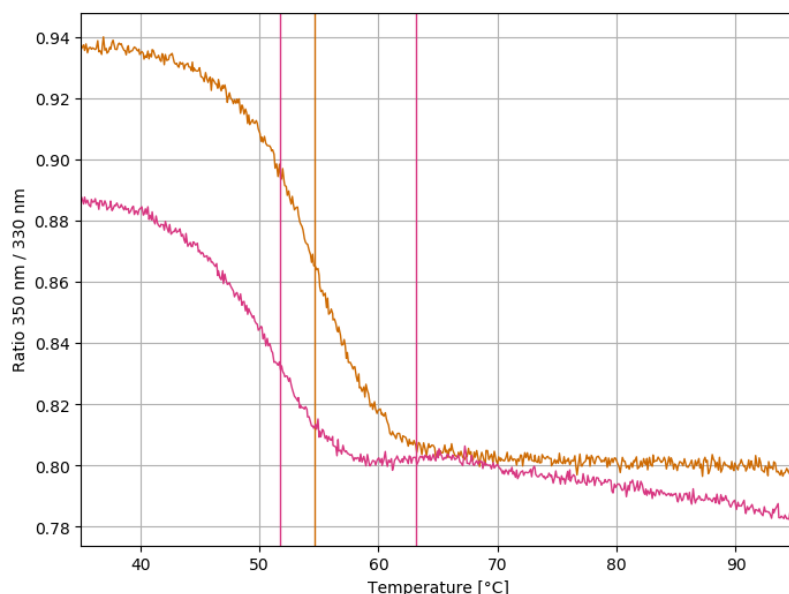


52 773 **Macromolecular interactions *in vitro*, comparing classical and innovative approaches training school.** Students  
53 774 during this training were able to use different approaches: Hydrodynamic (AUC-FDS, MST, SEC-MALS); Real time  
54 775 biosensor (BLI, switchSENSE) and Thermodynamic (ITC) to measure *in vitro* macromolecular interactions (protein-  
55 776 protein and protein-DNA). Experts on these approaches came from France and Europe as speakers and trainers.  
56 777 This event was made possible thanks to several networks and sponsors.

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778 **Figure S2. Unfolding profile plot of Ku70/Ku80.**

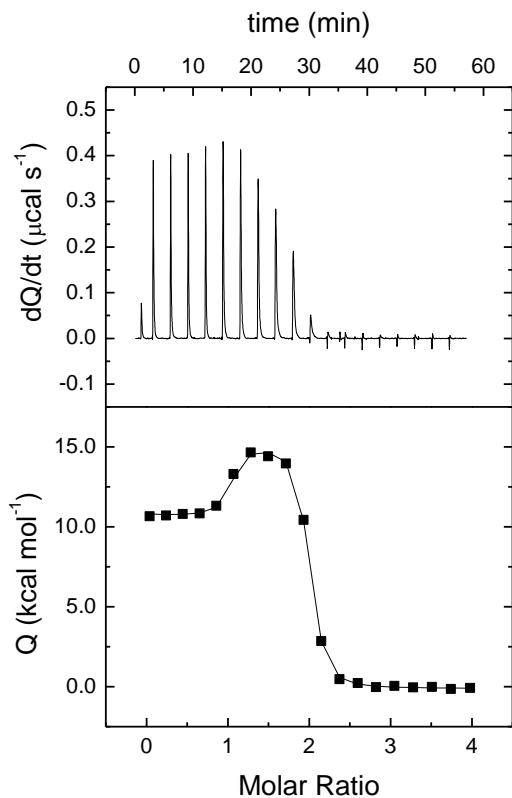


779 Quality and stability of samples are crucial to obtain accurate biophysical data. For all the biophysical studies  
780 shown in this manuscript, we used a full-length version of Ku (called KuFL), but for structural studies we used a  
781 shorter version where the C-terminus of each monomer is deleted (called KuCC). We took advantage of the new  
782 technology of *NanoTemper*, the Tycho NT.6 instrument, present during the training, to compare the thermal  
783 stability of these two versions of Ku protein by a fast measurement. Fluorescence intensity is recorded at 330 nm  
784 and 350 nm (emission profile of the Tryptophan residues). The brightness ratio 350 nm / 330 nm plotted against  
785 the temperature is called the unfolding profile plot and inflexion temperatures can be derived representing  
786 unfolding events. Tryptophan fluorescence of KuFL (orange) and KuCC (pink) were follow during a ramp of  
787 temperature of 35-95°C. KuFL showed a higher temperature of unfolding than KuCC (vertical bars). Thus, KuFL  
788 appears to be more stable than KuCC by a few degrees.



789 **Figure S3. Additional biophysical measurements of alphaRep interactions.**

**a ITC**

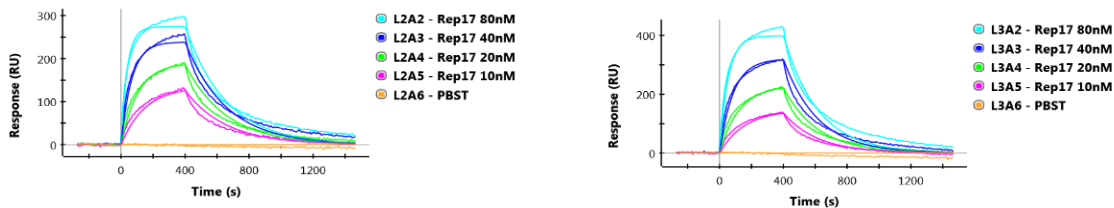


$K$ ( $\text{M}^{-1}$ )	$K_D$ (nM)	$\Delta H$ (kcal/mol)	$\alpha$	$\Delta h$ (kcal/mol)	$n$
$1.4 \cdot 10^9$	0.71	10.9	0.024	4.4	0.95

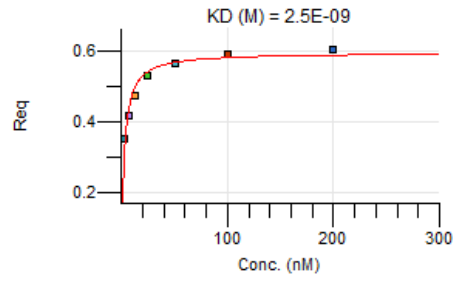
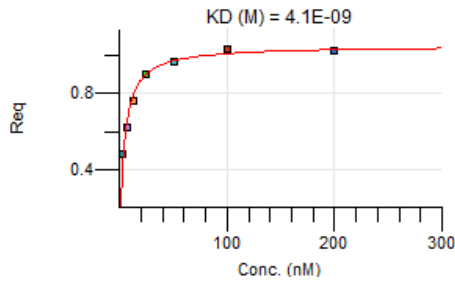
  

$K$ ( $\text{M}^{-1}$ )	$K_D$ (nM)	$\Delta H$ (kcal/mol)	$\alpha$	$\Delta h$ (kcal/mol)	$n$
$1.4 \cdot 10^9$	0.71	10.9	0.024	4.4	0.95

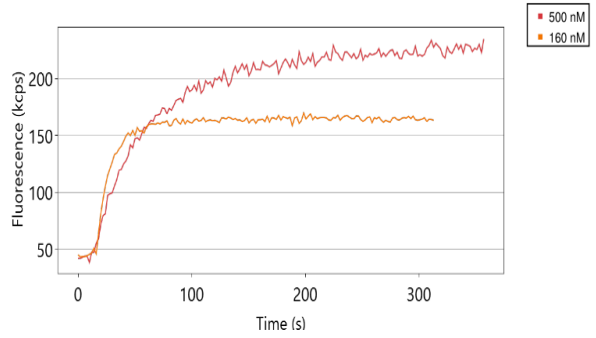
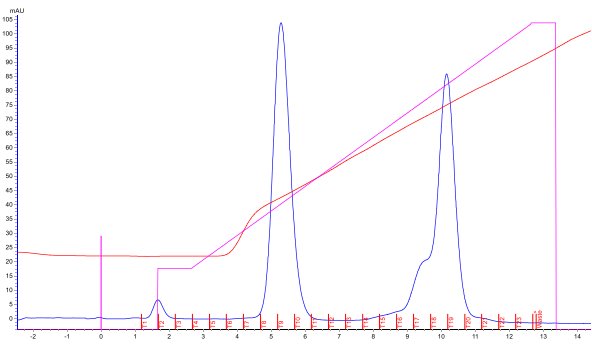
**b SPR**



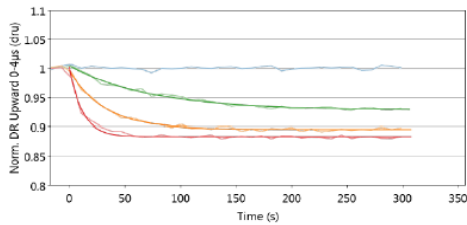
**c BLI**



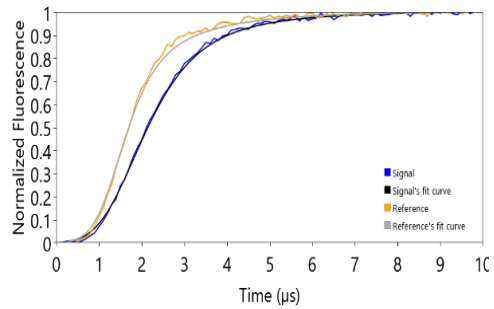
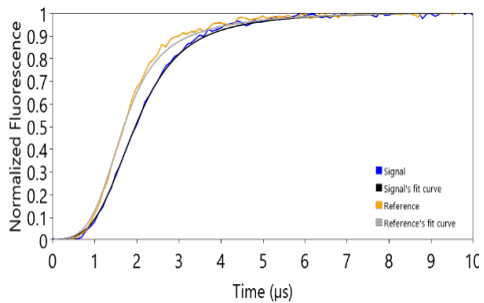
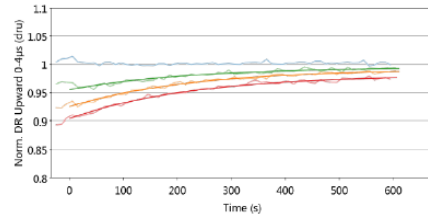
**d switchSENSE**



**Association**



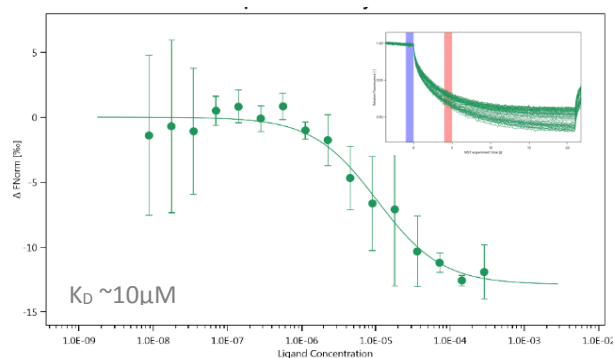
**Dissociation**



$D_h^{ave} = 3.02 \pm 0.05 \text{ nm} \text{ (n=8)}$

$D_h^{ave} = 3.82 \pm 0.09 \text{ nm} \text{ (n=4)}$

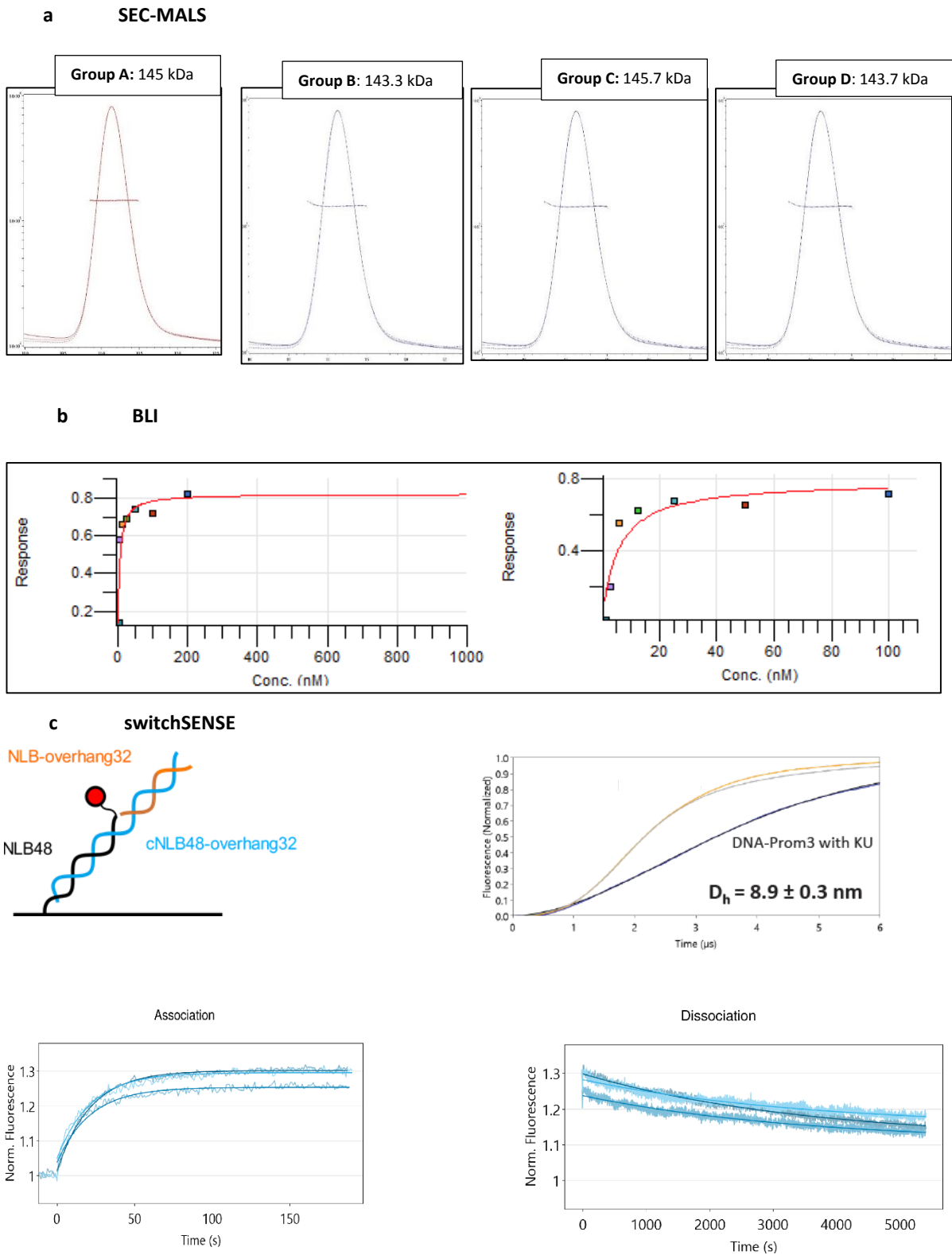
e MST



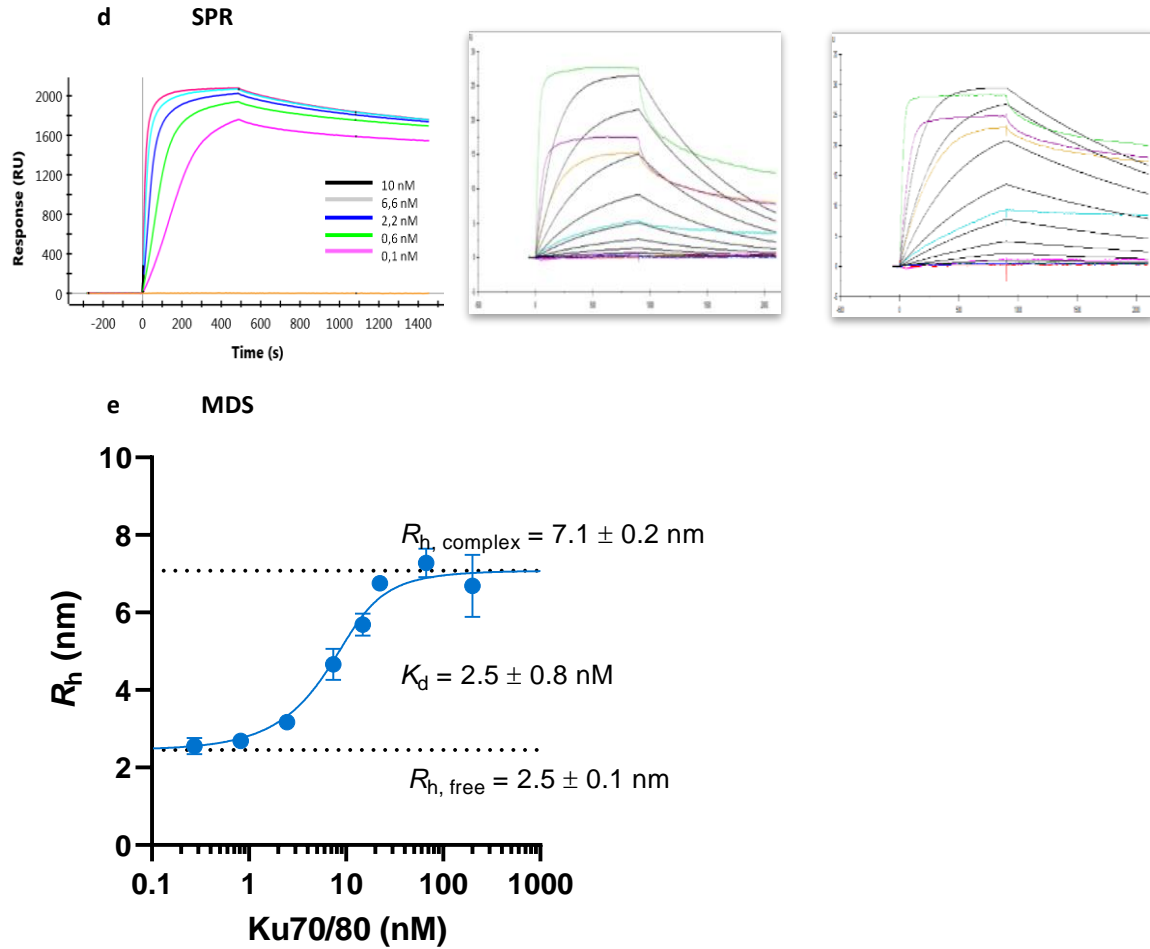
790 a) PEAQ-ITC data obtained before the training in our laboratory.  $K$ ,  $K_D$ , and  $\Delta H$  were the association constant, the  
791 dissociation constant, and the interaction enthalpy of either of the two binding sites when the dimer was  
792 unoccupied.  $\alpha$  and  $\Delta h$  were the cooperative interaction constant and the cooperative interaction enthalpy, which  
793 modulate the binding cooperative phenomenon (a factor that modulates the binding affinity and a term that  
794 modulates the binding enthalpy to the second site when there was a site already occupied, respectively).  $n$  was  
795 the active (or binding-competent) fraction of protein, since the stoichiometry was already included in the model.  
796  $K_{D1}$  and  $\Delta H_1$  were the intrinsic site-specific dissociation constant and binding enthalpy for the first binding site  
797 ( $K_{D1} = K_D$ ,  $\Delta H_1 = \Delta H$ ).  $K_{D2}$  and  $\Delta H_2$  were the intrinsic site-specific dissociation constant and binding enthalpy for  
798 the second binding site ( $K_{D2} = K_{D1} / \alpha$ ,  $\Delta H_2 = \Delta H_1 + \Delta h$ ). b) Sensograms of alphaRep A3-Rep17 interaction measured  
799 in duplicate on a ProteON XPR36 instrument from *Biorad*. For this experiment, association and dissociation times  
800 were 400 s and 1000 s, respectively with a flow rate of 50  $\mu L/min$  in phosphate buffered saline with 0.1 % Tween-  
801 20 (PBST) using His-Tag capturing (HTG) chip to immobilized A3 at 6.25 or 12.5  $\mu g/mL$  during 80 s (inducing 60  
802 RU and 95 RU, respectively). Rep17 ranges from 10 to 80 nM concentration. A  $k_{ON}$  of  $2.8 \cdot 10^{+5} M^{-1}s^{-1}$ , a  $k_{OFF}$  of  $3.9$   
803  $10^{-3} s^{-1}$  and a  $K_D$  of 13.8 nM were measured in the first run, and a  $k_{ON}$  of  $1.8 \cdot 10^{+5} M^{-1}s^{-1}$ , a  $k_{OFF}$  of  $4.6 \cdot 10^{-3} s^{-1}$  and a  
804  $K_D$  of 25.9 nM in the second one. c) Octet RED96e BLI steady-state analysis in duplicate. Colors code of Rep17  
805 concentrations: 3.13 nM (teal), 6.25 nM (purple), 12.5 nM (orange), 25 nM (green), 50 nM (cyan), 100 nM (red),  
806 200 nM (blue). D) Sample preparation for switchSENSE experiments. Anion-exchange chromatogram of the A3  
807 cross-linked protein (up, left). The cross-linked protein was the shoulder of the final peak that correspond to the  
808 free DNA. Hybridization of the cross-linked ssDNA-A3 on the chip, which carries a red fluorescent probe (up,  
809 right). Red profile corresponds to the control (free cNLB48 without protein) and the orange one to the cross-  
810 linked cNLB48-A3 conjugate. switchSENSE dynamic mode data of A3-Rep2 at three concentrations (middle, left).  
811 Normalized association (left) and dissociation (right) data were represented in function of time. switchSENSE  
812 sizing measurement of A3 hydrodynamic diameter ( $D_h$ ) (bottom, left), and of the A3-Rep17 complex (bottom,  
813 right). Measured signal curve is in blue (conjugated DNA), the associated fitted curve in blank, reference signal  
814 (cNLB48) is in orange, and the associated fitted curve in grey. d) MST analysis with blue fluorophore. Inset, MST  
815 traces, MST on used was 10 s (red band).

816 **Figure S4. Additional biophysical measurements of Ku-DNA interactions.**

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818 a) SEC-MALS elution profiles and molar masses of Ku alone in quadruple. b) Octet RED96e BLI steady-state data  
819 in duplicate. Colors code of Ku concentration: 3.13 nM (teal), 6.25 nM (purple), 12.5 nM (orange), 25 nM (green),  
820 50 nM (cyan), 100 nM (red), 200 nM (blue). c) switchSENSE measurement set up (top, left). By hybridization of  
821 complementary DNA carrying the target sequence as overhang, the surface is functionalized with the sequence  
822 of interest. switchSENSE static mode data of 1 Ku concentration in duplicate (bottom). switchSENSE sizing data  
823 (top, right). Reference (bare DNA) is depicted in yellow. DNA-protein-complex is depicted in blue. d) SPR  
824 sensograms by ProteON XPR36 of Ku-200 bp dsDNA interaction measured (right). A 200 bp biotinylated DNA was  
825 immobilized on a Streptavidin (NLC) chip. Ku70/Ku80 ranged from 0.1 to 10 nM concentrations, one heterodimer  
826 may induce 200 RU. A  $k_{\text{ON}}$  of  $7.8 \pm 4 \cdot 10^{+6} \text{ M}^{-1}\text{s}^{-1}$ , a  $k_{\text{OFF}}$  of  $1.5 \cdot 10^{-4} \text{ s}^{-1}$  and a  $K_D$  of  $7.3 \pm 3 \text{ nM}$  were measured. This  
827 experiment showed the threading of Ku on DNA, approximately 10 heterodimers bound to this 200 bp DNA. SPR  
828 sensograms by BIAcore T200 of Ku-42 bp dsDNA (middle) and, Ku-60 bp dsDNA (left) interaction measured by  
829 biotinylated DNA immobilized in a serie S sensor chip SA at 10 nM concentration. Same buffer as in switchSENSE  
830 experiment adding 0.2 mg/mL of BSA, flow rates of 20 to 100  $\mu\text{L}/\text{min}$ , and Ku at 5 nM as higher concentration for  
831 900 s of association and dissociation. A  $k_{\text{ON}}$  of  $1.0 \cdot 10^{+6} \text{ M}^{-1}\text{s}^{-1}$ , a  $k_{\text{OFF}}$  of  $11.7 \cdot 10^{-4} \text{ s}^{-1}$  and a  $K_D$  of 1.2 nM ( $R_{\text{max}}$  of 32.9  
832 RU) were measured for the 42 bp DNA and, a  $k_{\text{ON}}$  of  $1.4 \cdot 10^{+6} \text{ M}^{-1}\text{s}^{-1}$ , a  $k_{\text{OFF}}$  of  $5.0 \cdot 10^{-4} \text{ s}^{-1}$  and a  $K_D$  of 35.4 nM ( $R_{\text{max}}$   
833 of 31.6 RU) for the 60 bp DNA. Colors code of Ku concentrations: 5 nM (light green), 2.5 nM (purple), 1.25 nM  
834 (orange), 0.625 nM (cyan), 0.3125 (pink), 0.156 (dark green), 0.078 (blue), 0.039 (red). The differences observed  
835 in the results depend mainly on the setup strategy of the experiment. Nevertheless, open questions remain to  
836 answers: the purity of dsDNA and the stability of the protein will affect the results, but there is also the possibility  
837 of different ways of DNA fixation by Ku. e) In collaboration with *Fluidic Analytics*, we collected preliminary data  
838 to test their new instrument, Fluidity One-W with the Ku-DNA interaction. This is a novel technique in solution  
839 based in diffusional sizing of a complex in a microfluidic system. For this experiment we used the same 18 bp  
840 dsDNA-FAM as before (AUC, MST) at 10 nM concentration, and 1/3 dilution of Ku70/Ku80 from 200 to 0.09 nM  
841 concentration. We were able to measure  $R_h$  (DNA alone, complex) and  $K_D$  values.

842 **Tables**843 **Table S1. Summary of the results for the alphaRep proteins interaction using different**  
844 **techniques.**

	AUC-FDS	SEC-MALS*	ITC		MST	switchSENSE		BLI		SPR*
His-A3	Blue-NHS	Dimer	Monomer/high affinity	Dimer/low affinity	Red-His	Rep2*	Rep17	Monomer		Monomer
Ratio	1:1	1:1	1:1	1:1	N/A	1:1	1:1	N/A		N/A
K <sub>o</sub> (nM)	13.4 ± 1.8	N/A	0.6 ± 0.1	24 ± 8	8.0	0.29 ± 0.01	0.98 ± 0.16	3.2 ± 1.1	3.3 ± 0.8	3.97 ± 1.38
K <sub>ON</sub> (M <sup>-1</sup> s <sup>-1</sup> ) K <sub>OFF</sub> (s <sup>-1</sup> )	N/A	N/A	N/A	N/A	N/A	2.4 ± 0.1 10 <sup>6</sup> 6.9 ± 0.2 10 <sup>4</sup>	2.4 ± 0.2 10 <sup>6</sup> 2.3 ± 0.3 10 <sup>-3</sup>	2.6 ± 0.7 10 <sup>5</sup> 7.6 ± 0.7 10 <sup>-4</sup>	N/A	2.31 ± 1.79 10 <sup>5</sup> 4.28 ± 0.36 10 <sup>-3</sup>
Other information	S (A3) = 2.6 ± 0.1 S (A3/Rep17) = 3.0 ± 0.1	R <sub>h</sub> (A3) dimer = 3nm Mw (Rep2) = 11.6 kDa Mw (A3) = 44.8 kDa Mw (A3/Rep2) = 65.5 kDa	ΔH = 11.6 ± 0.5 kcal/mol	ΔH = 15.4 ± 0.7 kcal/mol	N/A	D <sub>h</sub> (A3) monomer = 3 nm D <sub>h</sub> (A3-Rep2) = 3.8 nm	N/A	N/A	N/A	N/A

845 In the last column in grey, previous SPR data obtained by P. Minard's team. \*Rep2

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847 **Table S2. Summary of the results obtained for the Ku-DNA interaction using the different**  
848 **techniques.**

	AUC-FDS	ITC		SEC-MALS	BLI		MST	switch SENSE	SPR	Diffusional sizing
<b>DNA(bp)</b>	<b>18*</b>	<b>18</b>	<b>42</b>	<b>42</b>	<b>42**</b>		<b>18*</b>	<b>48</b>	<b>200**</b>	<b>18*</b>
<b>Ratio Ku vs DNA</b>	1:1	1:1	2:1	2:1, 1:1	N/A		N/A	1:1	20:1	1:1
<b>K<sub>D</sub> (nM)</b>	12.9 ± 3.2	3.5 ± 0.8	3.6 ± 0.4	N/A	0.4 ± 0.1	5.2 ± 0.2	2.8	1.0 ± 0.2	7.3 ± 3.0	2.5
<b>k<sub>ON</sub> (M<sup>-1</sup>s<sup>-1</sup>)</b> <b>k<sub>OFF</sub> (s<sup>-1</sup>)</b>	N/A	N/A	N/A	N/A	1.9 ± 0.1 10 <sup>+6</sup> 7.2 ± 0.3 10 <sup>-4</sup>	N/A	N/A	2.6 ± 0.2 10 <sup>+6</sup> 2.3 ± 0.3 10 <sup>-4</sup>	7.8 ± 4.0 10 <sup>+6</sup> 1.5 ± 0.8 10 <sup>-4</sup>	N/A
<b>Other information</b>	S (DNA) = 2.2 ± 0.1 S (1:1) = 7.3 ± 0.2	5.1 ± 0.1 kcal/mol	23.9 ± 4.9 kcal/mol	Mw (DNA) = 26.1 kDa Mw (Ku) = 144.4 kDa Mw (1:1) = 171.0 kDa Mw (2:1) = 306.2 kDa	N/A	N/A	N/A	D <sub>h</sub> = 8.9nm	N/A	R <sub>h</sub> = 7.1nm

849 The last two columns came from additional data: Preliminary SPR obtained by Charbonnier's team, and MDF in  
850 collaboration with Fluidic Analytics. DNA modified \*FAM or \*\*Biotin.

## 1 **Macromolecular interactions *in vitro*, comparing classical and novel approaches**

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4 Quétard<sup>13</sup>, Christine Ebel<sup>14</sup>, Alain Roussel<sup>15</sup>, Jean-Baptiste Charbonnier<sup>1</sup>, Paloma Fernández  
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## 25 **Abstract**

26 Biophysical quantification of protein interactions is central to unveil molecular mechanisms of  
27 cellular processes. Researchers can choose from a wide panel of biophysical methods,  
28 including classical and more novel ones. A real-life proof-of-concept was carried out during an  
29 ARBRE-MOBIEU training school held in June 2019 in Gif-sur-Yvette, France  
30 (<https://mosbio.sciencesconf.org/>). Twenty European students benefited from a one-week  
31 training with lessons and practical sessions on six complementary approaches: (i) Analytical  
32 UltraCentrifugation with or without a Fluorescence Detector System (AUC-FDS), (ii) Isothermal  
33 Titration Calorimetry (ITC), (iii) Size Exclusion Chromatography coupled to Multi-Angle Light  
34 Scattering (SEC-MALS), (iv) Bio-Layer Interferometry (BLI), (v) MicroScale Thermophoresis  
35 (MST) and, (vi) switchSENSE. They implemented all these methods on two examples of  
36 macromolecular interactions: firstly, a protein-protein interaction between an artificial  
37 alphaRep binder, and its target protein, also an alphaRep; secondly, a protein-DNA interaction



38 between a DNA repair complex, Ku70/Ku80 (hereafter called Ku), and its cognate DNA ligand.  
39 The students acknowledged that the workshop provided them with a clearer understanding  
40 of the advantages and limitations of the different techniques and will help them in the future  
41 to choose the approaches that are most relevant or informative ~~to~~for their projects.

## 42 **Keywords**

43 Molecular scale biophysics, macromolecular interactions, artificial binders, double-stranded  
44 DNA breaks repair factors

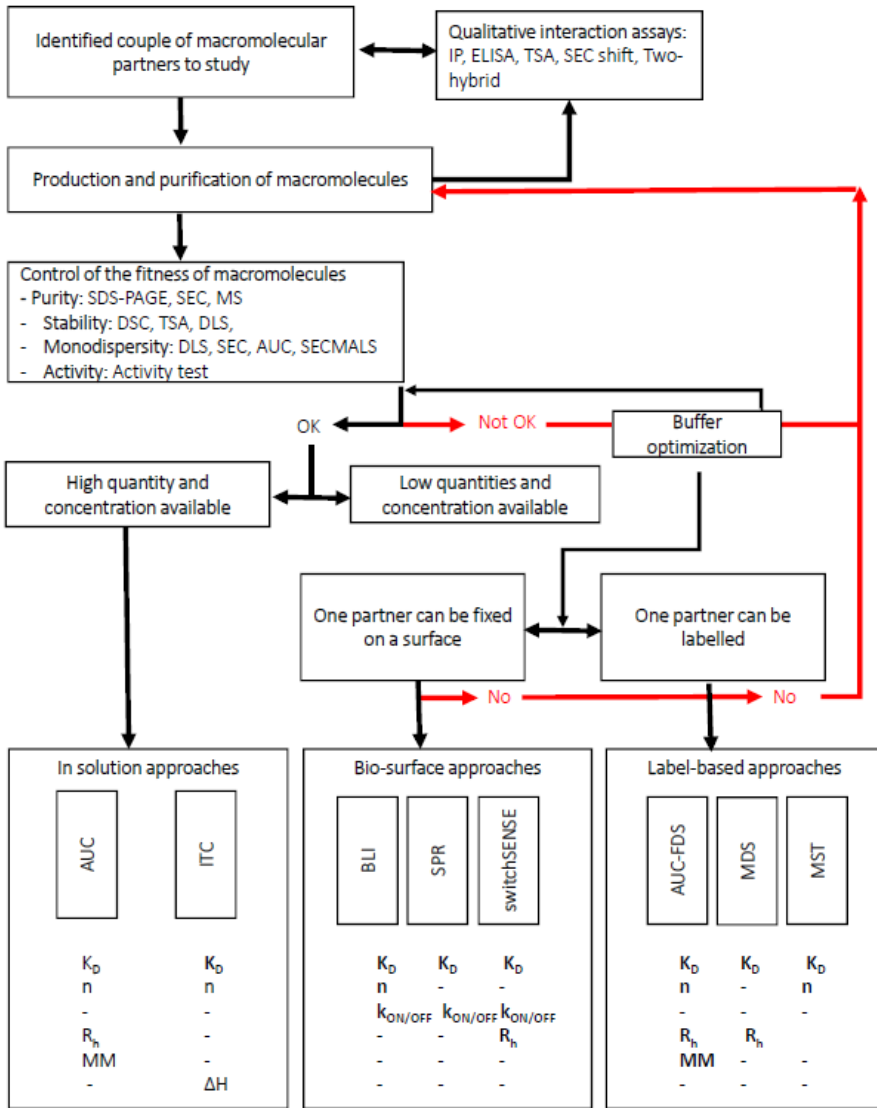
## 45 **Introduction**

46 Macromolecular interactions play a central role in the activation/inactivation of most cellular  
47 mechanisms. These interactions can be measured *in cellulo*, or *in vitro*, and predicted *in silico*.  
48 The classical *in cellulo* methods (such as tap-tag or two-hybrid) allow large-scale studies, but  
49 in order ~~to the characterization of confirm that~~ a direct interaction between two  
50 macromolecules occurs/relies on the ~~between two macromolecules~~, quantitative *in vitro*  
51 measurements ~~are needed~~. These measurements allow to characterize interactions not only  
52 in terms of affinity, but also to determine additional kinetic and thermodynamic parameters,  
53 as well as to define the hydrophobic or hydrophilic nature of the interface. They also give  
54 access to the stoichiometry of the assembly and allow to map the regions involved by using  
55 different constructs or mutants. *In vitro* measurements can also be useful to evaluate the the  
56 drugability of the interaction site or to understand the role of ~~post-translational modifications~~  
57 ~~or other~~ endogenous regulatory events on the formation of complexes (post-translational  
58 modifications). These measurements are essential to set-up predictive ~~By~~ *in silico* docking  
59 ~~analysis tools. Conversely, by in silico analysis, we can predict~~ the structure of macromolecular  
60 complexes ~~or as well as~~ the impact of functional substitutions can be predicted, helping to  
61 optimize experimental design (Andreani and Guerois, 2014).

62 The original idea of the project presented here originated during the organization of a  
63 European Training School in Molecular Scale Biophysics (<https://mosbio.sciencesconf.org/>)  
64 within the MOBIEU COST Action. We proposed to the participants to compare different  
65 techniques allowing to measure macromolecular interactions *in vitro*. Nowadays, there is a  
66 large panel of possibilities, and it becomes difficult to choose which technology will be the

67 best adapted when embarking into a new project. Each approach presents advantages and  
68 drawbacks, and it is therefore difficult for the user to choose from the beginning which one  
69 will be most adapted to the properties of the interaction partners. In the workshop, we  
70 focused on six approaches (Fig. 1). Choosing between the different techniques can be  
71 considered in a progressive manner. If none of the interaction partners can be easily labeled  
72 or immobilized on a surface, approaches in which ~~the macromolecules~~both partners are in  
73 solution (AUC, ITC, and SEC-MALS) should be favored (Fig. 1, top). However, several of them  
74 require large quantities of biological material. When a partner can be immobilized easily on a  
75 bio-surface, without affecting its function, approaches like BLI, switchSENSE and Surface  
76 Plasmon Resonance (SPR) will be often tested, since they offer the possibility to use small  
77 amounts of the immobilized partner (called ligand) (Fig. 1, middle). Finally, when the partners  
78 can be labeled, again without affecting their function, fluorescent probes can be grafted  
79 allowing the use of reduced amounts of material and facilitated signal analysis (AUC FDS, MST,  
80 or Microfluidic diffusional sizing (MDS)) (Fig. 1, bottom).The choice of the optimal approach  
81 may further rely on additional criteria including the solubility of the partners, the instrument  
82 environment or non-specific interactions with instrument surfaces. Finally, it should be  
83 stressed that it is preferable to perform measurements using several orthogonal techniques  
84 to fully validate and characterize a biological interaction, and specify its features, such as  
85 stoichiometry, kinetics or thermodynamics.

86 **Figure 1. Decisional tree to help choosing the biophysical approach ~~that~~ best suited for the**  
87 **study of a specific molecular interaction.**





102 Detector System); Microfluidic Diffusional Sizing (MDS); MicroScale Thermophoresis (MST) (bottom, right).  
103 These six approaches give access to different parameters of the interaction and present some specific  
104 limitations. If a high quality sample (pure, stable, monodisperse) is available in large quantity (up to mg amount)  
105 one may start with label-free and in solution approaches. Otherwise, if material is limited for one partner, surface  
106 approaches and labeling approaches are a good alternative. Finally, when labeling is possible, MST and AUC-FDS  
107 are and highly complementary approaches to cross-validate interactions measurements. Surface approaches  
108 are best candidates to get access to the kinetic parameters of interaction. Six of these approaches were used in  
109 the training school. The six approaches give access to different parameters of the interaction and present some  
110 specific limitations. AUC: Analytical Ultracentrifugation; ITC: Isothermal Titration Calorimetry, SEC-MALS: Size  
111 Exclusion Chromatography with Multi-Angle Light Scattering; BLI: Bio Layer Interferometry; MST: MicroScale  
112 Thermophoresis; AUC-FDS: Analytical Ultracentrifugation with a Fluorescence Detector System; S: Sedimentation  
113 coefficient;  $K_D$ : Dissociation constant ( $K_D$ );  $n$ : stoichiometry ( $n$ ); association and dissociation rates ( $k_{ON}$ ,  $k_{OFF}$ );  $D_h$ ,  
114 hydrodynamic radius/diameter or  $\langle R_h \rangle$ , hydrodynamic radius;  $k_{ON}$ ,  $k_{OFF}$ ; association and dissociation rates;  $M$ ,  
115 mass transport; Molecular mass (MM).

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116 In this training school, we used as examples two different macromolecular interactions  
117 systems, that have been well characterized in our laboratories using several of the approaches  
118 discussed here. One is an interaction between two proteins, and the other a protein-DNA  
119 interaction, both with an affinity in the nanomolar range. Reference data were initially  
120 produced in our laboratories. A group of 20 participants reproduced our measurements during  
121 the five-day MoSBio Training School.

122 The first project comes from P. Minard's team, who uses an original family of artificial  
123 helicoidal repeat proteins, called alphaRep (Guellouz et al., 2013). AlphaRep libraries allow to  
124 select tight binders against a variety of targets by phage display. The alphaRep's are highly  
125 soluble proteins, easily expressed in *E. coli*, which display a very high thermal stability. These  
126 proteins are cysteine-free and, thus do not contain disulfide bonds. They are composed by  
127 repeated motifs made with two antiparallel alpha helices. Clusters of variable side chains,  
128 mainly in the second helix, are positioned on the same face of the motifs. The ensemble of all  
129 these variable motifs forms a library of surfaces from which tight binders can be extracted  
130 against a given target. The alphaRep's have been used for several applications, such as  
131 chaperones for crystallization and structural studies of difficult targets (Valerio-Lepiniec et al.,  
132 2015; Di Meo et al., 2017; Chevrel et al., 2018; Campanacci et al., 2019), as well as in  
133 biophysical and live cell ~~applications-studies~~ (Léger et al., 2019; Prasad et al., 2019; Fernandez  
134 et al., 2020; Léger et al., 2020). New applications of these artificial binders are currently ~~being~~  
135 explored in relation ~~with~~ their ability to be expressed in eukaryotic cells. Here we analyzed  
136 the interactions between two alphaRep's (Rep2 and Rep17) selected against a protein target,  
137 (A3), which is itself an alphaRep. The interaction between the alphaReps has been extensively  
138 characterized before in the laboratory by ITC, SEC-MALS and AUC, but also by Circular  
139 Dichroism (CD), SPR and Fluorescence Resonance energy Transfer (FRET) (not used in this  
140 training school) (Guellouz et al., 2013; Di Meo et al., 2017; Léger et al., 2019). Both A3 / Rep2  
141 and A3 / Rep17 interactions were tested previously to determine which one was the most  
142 appropriate for the training. ~~Because, and~~ the former is weaker than the latter, ~~we focused~~  
143 ~~only on the only A3 / Rep17 interaction.~~ ~~During the training only A3 / Rep17 interaction was~~  
144 ~~measured.~~

145 The second project comes from J.B. Charbonnier's team. It concerns proteins involved in DNA  
146 repair, and more precisely, in the classical Non-Homologous End Joining (c-NHEJ), the main

147 Double-Strand Break (DSB) repair pathway in human. The Ku70/Ku80 (Ku) heterodimer plays  
148 a central role in the recognition of DSB ends, as it is the first repair factor that interacts with  
149 them. Ku binds tightly to DNA ends in a sequence independent manner thanks to its ring-  
150 shaped structure (Walker et al., 2001). Ku then iteratively recruits different NHEJ partners  
151 (ligase 4, nucleases and polymerases) (Chang et al., 2017; Frit et al., 2019). Ku also contributes  
152 to the tethering (synapse) between the two DSB ends to avoid misrepair with other DSB ends.  
153 J.B. Charbonnier's team (Tadi et al., 2016; Nemoz et al., 2018) has recently described the  
154 recruitment mechanism of some NHEJ factors by Ku at the molecular level. DSBs, despite being  
155 deleterious DNA lesions, are generated on purpose during radiotherapy or in genome editing  
156 by CRISPR-Cas9. Understanding the molecular basis of the c-NHEJ is thus central to improve  
157 these major biotechnological applications. Here we analyzed the interaction between Ku and  
158 DNA substrates of different lengths, to determine the most appropriate ~~for~~ the training and  
159 to study the threading of Ku on DNA. The characterization of the interaction between Ku and  
160 DNA ~~has been~~ extensively studied in the laboratory by ITC, MST (Tadi et al., 2016; Nemoz et  
161 al., 2018; Gontier ~~et al., 2021, Chapter of MiMB, in press~~), switchSENSE, AUC, BLI (data not  
162 published) and, more recently by other techniques not used during the training school (SPR,  
163 MDS or FRET). One Ku occupies about 18 bp ~~on DNA. And~~ several Ku molecules can thread  
164 on DNA when ~~the its size of the DNA~~ is longer than 18bp, ~~and a long DNA can be covered~~  
165 ~~by~~with one Ku molecule every 18bp.

166 We present in this article the results obtained by 20 students during a European Training  
167 School in Molecular Scale Biophysics that took place from June 3<sup>rd</sup> to 7<sup>th</sup>, 2019 at I2BC at Gif-  
168 sur-Yvette, France. We report the protocols used to analyze the two systems under study  
169 (protein/protein and protein/DNA interactions) using the six biophysical methods mentioned  
170 above. The results obtained by the students during ~~this the one-week~~ ~~course~~ are shown and  
171 compared to our previous published data when available. Finally, we compare the advantages  
172 and drawbacks of the different approaches used during this training ~~school~~ and present some  
173 feedbacks from the students, allowing to have a global overview of the pros and cons of these  
174 six complementary biophysical approaches.

175 **Materials and Methods**

176 **Biophysical approaches in solution that do not require labeling of an interaction partner**

177 **Size Exclusion Chromatography with Multi-Angle Laser Light Scattering detection (SEC-**

178 **MALS)**: SEC-MALS allows to determine the absolute molar mass of the components ~~of a~~  
179 ~~protein/multiprotein~~ present in a macromolecular sample. It indicates if elution peaks are  
180 homogenous ~~in term of protein composition~~ or if they are composed of mixtures, either of  
181 different oligomers or of different conformers. SEC-MALS allows size determination if an  
182 online Dynamic Light Scattering (DLS) detector is included in the setup (Folta-Stogniew, 2006).

183 In order to determine stoichiometry in a macromolecular hetero-complex, differences in  
184 extinction coefficients are required. An experimental determination of extinction coefficient  
185 by analyzing each partner separately, will be more precise than *in-silico* prediction. For a more  
186 precise estimation of molar mass of complex components, you need to know the refractive  
187 index increment (dn/dc value) of each complex partner at the wavelength of the MALS system  
188 laser. We used an HPLC system from *Shimadzu* coupled to a MALS detector (miniDAWN  
189 TREOS) plus a DLS detector (QELS), and a refractometer (optilab T-rEX) from *Wyatt*  
190 ~~technologies (Fig. S1)~~. To run an experiment, one needs 1 L of running buffer to equilibrate  
191 the column and detectors, ~~and from~~ 2 mg/mL of protein (for MW of 20 kDa), down to 0.5  
192 mg/mL [if the MW is higher than 150 kDa]. Depending ~~of on~~ the column used, the volume of  
193 the sample (30 µL for Bio-SEC-3 *Agilent*, 50 µL for KW-803/804 *Shodex*, and 100 µL for  
194 Superose 6, Superdex 75 or Superdex 200 increase 10/300 GL *Cytiva*) and the time of elution  
195 can differ. A run (including an equilibration step and a control BSA sample) takes typically a  
196 few hours and consumes ~~about~~ 40-200 µg of sample.

197 **Isothermal Titration Calorimetry (ITC)**: ITC allows the direct, and thorough thermodynamic  
198 characterization of interactions between molecules in solution with no limitation of partner  
199 size and without labeling (Holdgate, 2001; Krell, 2008; Velazquez-Campoy and Freire, 2006)  
200 ~~and without labeling~~. ITC ~~is an equilibrium solution technique~~ allows to quantify dissociation  
201 constants ( $K_D$ ) ~~values~~, but also other interaction parameters (enthalpy, entropy,  
202 stoichiometry, and heat capacity). ITC is not affected by the optical properties of the samples,  
203 but may be very sensitive to the composition of the buffer (e.g. ~~presence of DMSO or in~~  
204 particular to mismatches between partner sample solutions). In a microcalorimeter the two  
205 cells (reference and sample) needs to be kept at exactly the same temperature. A heat sensing

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206 device detects temperature difference between the cells when binding occurs, upon serial  
207 injection of a ligand, and give feedback to the heaters, which compensate for this difference  
208 and return the cells to equal temperature. Each injection gives then rise to a peak of emitted  
209 or absorbed heat whose surface is proportional to the amount of binding. For the ITC  
210 experiments, we used three instrument models: VP-ITC, ITC200 and PEAQ-ITC all from  
211 *Malvern Panalytical* (~~Fig. S1~~). One of the interacting partners is placed in a cell (1.4 mL for the  
212 VP-ITC, 200  $\mu$ L for the ITC200 and PEAQ-ITC) and the other in a syringe (300  $\mu$ L for the VP-ITC,  
213 40  $\mu$ L for the ITC200 and PEAQ-ITC). Sequential injections are made from ~~a the~~ syringe (5-10  
214  $\mu$ L for the VP-ITC, 1-2  $\mu$ L for the ITC200 and PEAQ-ITC) into the cell. ~~To produce~~ the data  
215 ~~described-reported~~ here, we used 80-990  $\mu$ g of the molecule in the cell, and 130-320  $\mu$ g of the  
216 molecule in the syringe. The transient heat effect due to complex formation (and other  
217 potential unspecific phenomena) upon partner injection is measured as the titration  
218 progresses, from which the binding isotherm is constructed.

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#### 219 **Biophysical approaches with a partner immobilized on a surface**

220 **Bio-Layer Interferometry (BLI):** BLI is a fast, high throughput and label-free technology for  
221 measuring biomolecular interactions by analyzing the interference pattern of white light  
222 reflected from a layer of immobilized macromolecules on a biosensor tip ~~and versus an~~  
223 internal reference (Abdiche et al., 2018). It enables real-time ~~analysis for~~ determination of  
224 affinity, kinetic parameters and concentration, with one of the binding partners immobilized  
225 onto the biosensor surface (ligand) and the other in solution (analyte). This microfluidic-free  
226 technology is particularly adapted for performing binding assays in crude lysates or cell culture  
227 media. We used an Octet RED96e during the training, and ~~previously an~~ RED384 during the  
228 preparation phase, both from *FortéBio* (~~Fig. S1~~). In all cases, one of the interaction partners  
229 will be non-covalently captured on a glass sensor probe. The sensor will then be incubated in  
230 microplate wells containing the second interaction partner in solution. A light beam is sent  
231 through the sensor, and the reflected beam is analyzed using an interferometric detector, that  
232 measures the spectral shift due to the variations of density (optical thickness) that the bilayer  
233 undergoes upon association and dissociation of the complex. The real-time monitoring of  
234 these variations allows to record a « sensorgram », from which the kinetic rates ( $k_{ON}$  and  $k_{OFF}$ ),  
235 as well as the equilibrium constant  $K_D$  can be determined through mathematical curve fitting.  
236 We used 1-5  $\mu$ g/mL of ligand to load NTA sensors for 20-120  $s_z$  for low density $_z$  or 120-600  $s_z$

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237 for high density, and a range of at least 7 (2-fold dilutions (starting at 100-200 nM) of the  
238 analyte with an association time of 600-900 s. We consumed ~~per run~~ about 8 µg of protein A3,  
239 and 400 ng of 42 bp biotinylated DNA ~~per run~~ for the immobilization, and about 2-14 µg of ~~the~~  
240 respective analytes. The concentration of ligand could be reduced (at least by 2) in favor of a  
241 longer incubation. The consumption of the analyte, depends on the affinity of the interaction,  
242 as the concentrations used should range from  $K_D / 120$  to  $10 \times K_D$ .

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243 **switchSENSE**: switchSENSE technology is based on short DNA nanolevers (48 bp in our case),  
244 which are immobilized on gold electrodes in a microfluidic channel. The intrinsically negatively  
245 charged DNA nanolevers can be electrically actuated (“switched”) on the gold surface to  
246 oscillate at high frequencies (Knezevic et al., 2012). A switchSENSE microfluidic biochip  
247 contains four flow channels, each containing six gold electrodes. Switching of the DNA is  
248 mediated by alternating the voltage across the gold surface. The motion of the levers is  
249 tracked in real time (µs scale) via electrically triggered time correlated single photon counting

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250 (E-TCSPC) detecting a fluorescent probe (dye) present on top of the immobilized DNA strands.  
251 The recorded fluorescence intensity correlates to the orientation of the DNA nanolever  
252 relative to the surface as the fluorescence is gradually quenched upon approaching the gold  
253 electrode due to energy transfer. ~~time-resolved single photon counting detecting a~~  
254 fluorescent probe present on the immobilized DNA strands. The complementary DNA strands

255 can be cross-linked to a ligand via amine or thiol coupling or click-chemistry. By hybridization  
256 of this conjugated complementary strand to the surface-tethered DNA nanolever, the surface  
257 is functionalized with the ligand of interest. Upon binding of an analyte, the hydrodynamic  
258 friction of the levers is affected-increased and subsequently the movement of the levers is  
259 slowed down. This change in switching speed is used by the system to determine the size ( $D_h$ )  
260 or conformational changes of ligands and complexes. The kinetics of molecular interactions  
261 ( $k_{ON}$ ,  $k_{OFF}$ ,  $K_D$ ) can be followed-monitored using two measurement modes: dynamic or static.

262 In the first case, analyte binding is measured through the change of the oscillation rate of the  
263 electrically actuated DNA nanolevers (changes in dynamic response). In the second case, the  
264 DNA nanolevers are kept at an upright position, in a constant electric field, and analyte in close  
265 proximity to a dye can alter the-its local chemical environment resulting in a fluorescence  
266 change (also called Fluorescence Proximity Sensing). Binding is then measured thanks to the  
267 fluorescence intensity variation of the functionalized nanolever. For the switchSENSE

268 experiments, we used a DRX<sup>2</sup> device from *Dynamic Biosensors* with two LED light sources (for  
269 the excitation of red and green fluorophores) ~~(Fig. S1)~~. In all cases, a hundred µg of protein  
270 where enough to generate cross-linked complementary strands (cNL-DNA) for several round  
271 of experiments, since one measurement needs only 40 µL of 100 nM DNA-protein conjugate.  
272 A sizing measurement classically takes less than an hour. The ~~amount-quantity~~ of analyte  
273 needed for a kinetic experiment depends on the overall affinity, ~~which delimits the~~ flow rates,  
274 and ~~the association/dissociation times to be used. During association, a~~ too slow flow rate  
275 can ~~be the cause for result in~~ mass transport limitation ~~effect and~~ during ~~adi~~dissociation and  
276 ~~inadequate flow rate can result~~ in re-binding effects ~~during dissociation~~. Here we used flow  
277 rates of 100-500 µL/min, association times of 80-300 s, and 1500 s of dissociation time. ~~W~~  
278 ~~sample quantities, w~~We used 150 ng of the cross-linked ligands, and 2-20 µg of its partner for  
279 a series of 3 concentrations.

## 280 Biophysical approaches with a labeled partner

281 **Analytical UltraCentrifugation with Fluorescence Detection (AUC-FDS):** AUC is a powerful  
282 technique for the characterization of macromolecules and macromolecular self- and hetero-  
283 association processes in solution. It was used here with labeled protein or DNA, but it can be  
284 used with non-labeled material like SEC-MALS and ITC. An analytical ultracentrifuge is a high-  
285 speed centrifuge equipped with one or more detectors (absorbance/interference,  
286 fluorescence) allowing to monitor sedimentation in real time. Two types of complementary  
287 experiments can be performed, sedimentation velocity and sedimentation equilibrium (Zhao  
288 et al., 2013). Sedimentation velocity experiments allow to determine the size distribution of  
289 species, their aggregation and oligomerization, sedimentation coefficients, hydrodynamic  
290 radius, shape and molar masses, their stoichiometry and  $K_D$  (by isotherm fitting ~~of~~  
291 sedimentation coefficients measured at various concentrations). Sedimentation equilibrium  
292 ~~is experiments are~~ suited for well-defined samples, and gives molar mass and  $K_D$  information.  
293 The centrifugation speed can be set between 600 and 260 000 g allowing to study all sizes of  
294 macromolecules. The duration of sedimentation experiments ranges from 2 h to several days  
295 ~~(Fig. S1)~~. ~~dn/dc and UV extinction coefficients values, which are needed to analyse~~  
296 ~~data obtained with absorbance and interference optics, can be~~  
297 ~~determined from the protein sequence using the SEDFIT calculator module (Schuck, 2000)~~. For  
298 the AUC-FDS experiments we used a ProteomeLab XL-I ultracentrifuge from *Beckman Coulter*

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299 equipped with a Fluorescence Detection System (FDS) from AVIV Instruments ~~(Fig. S1)~~. A  
300 sedimentation velocity experiment requires 100-450  $\mu$ L of sample at 0.3 to 1.5 OD, for  
301 absorbance/interference detection, and 5-60 nM concentration, for fluorescence detection,  
302 whereas sedimentation equilibrium requires 130  $\mu$ L at 0.2 to 0.5 OD. In interference, the limit  
303 of detection is 0.1 mg/mL, but in absorbance it depends on the sample extinction coefficients.

304 To characterize an interaction using the AUC fluorescence  
305 detector, a range of 2 nM to 2  $\mu$ M is typically used. When the  
306 affinity is very high, a range between 2 pM and 2 nM is chosen, but a  
307 blocking agent - e.g. BSA at 1 mg / mL - is added in the sample to avoid  
308 non-specific adsorption and loss of fluorescent dye. To produce the data described here, we  
309 used about 1-3.5  $\mu$ g of the fluorescent molecule and 8-80  $\mu$ g of the non-fluorescent molecule.

310 **Microscale Thermophoresis (MST):** MST is a novel technology for the analysis of biomolecules  
311 based on the modification of fluorescence intensity induced by the temperature, due to  
312 Temperature Related Intensity Changes (TRIC) and the directed movement of particles in a  
313 microscopic temperature gradient (thermophoresis) (Asmari et al., 2018; Jerabek-Willemsen  
314 et al., 2011). Thermophoresis is influenced by a combination of changes at the level of the  
315 hydration shell, shape, charge..., of biomolecules (all the parameters that influence the Soret  
316 coefficient) of biomolecules, which result in differences of movement along the temperature  
317 gradient as well as the of brightness of the fluorescent tag. MST provides information on the  
318 binding affinities with good accuracy and sensitivity in the pM to mM range. This technology  
319 allows immobilization-free measurement of interactions in any buffer and complex biological  
320 liquid, but requires one of the two partners to be labeled with a fluorescent dye to measure  
321 protein-protein interactions (there is also a label free instrument that detects the intrinsic  
322 fluorescence of proteins). Any size of unlabeled molecules can be used (from ions, to large  
323 proteins). MST experiments are performed in capillaries, thus require a low sample  
324 consumption. Method development for MST is usually fast and consists of three steps: (i)  
325 ensure there is enough fluorescence signal intensity. (ii) Check your sample for sticking to the  
326 capillaries. (iii) Establish the noise floor of your experiment by measuring MST of just your  
327 target without ligand at the final settings (final concentration, excitation laser and 'MST  
328 power' = IR laser LED power to be used). We used a Monolith NT.115 blue/green and a  
329 red/blue from NanoTemper Technologies, (Fig. S1). MST experiments, performed in

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330 ~~capillaries, require a low sample consumption~~ (200  $\mu$ L at 20 nM of the fluorescent molecule,  
331 and 20  $\mu$ L of the non-fluorescent molecule at the highest concentration needed, ~~(~~depending  
332 on the expected  $K_D$ ). To produce the data described here, we used about 50-350 ng of the  
333 fluorescent molecule and 6-12  $\mu$ g of the non-fluorescent molecule.

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#### 334 **Sample preparation**

335 ~~To perform biophysical characterization measurements, samples of high purity, stability and~~  
336 ~~monodispersity are needed (Fig. S1). We chose well characterized systems from our~~  
337 ~~laboratories, and the concentration ranges employed to accurately determine binding~~  
338 ~~parameters were already known.~~

339 AlphaReps are recombinant proteins produced by standard overexpression procedures in  
340 *E.coli* (Guellouz et al., 2013). For our experiments, we used the following alphaRep proteins:  
341 A3, which is the target and Rep17 and Rep2, which are the binders. Because A3 forms  
342 homodimers at high concentration, it was used as ligand for real-time biosensors approaches  
343 (BLI, switchSENSE) and was the labeled partner in AUC-FDS and MST experiments. In the  
344 conditions used, we assumed A3 is a monomer, ~~as t.~~ The simultaneous presence of the  
345 monomeric and dimeric forms would make the analysis difficult. ~~A3 dimers are unable to~~  
346 ~~interact with Rep17, and it is only when they dissociate into monomers that, the interaction~~  
347 ~~with Rep17 occurs. A dissociation constant of 37 nM for the A3 / A3 dimer was measured by~~  
348 ~~AUC (Léger et al., 2019). This is why we observe several events in ITC: the dissociation of the~~  
349 ~~A3 dimer and the interaction with Rep17.~~

350 Ku is a recombinant protein produced by standard overexpression procedures in insect cells  
351 (Nemoz et al., 2018). For our experiments, we used different lengths of dsDNA depending on  
352 the assay format. Shorter DNA are not adapted for biosensor approaches, since a DNA too  
353 close to the surfaces will hinder its interaction with Ku. DNA is practical to work with, because  
354 it is easy to modify and is commercially available. For immobilization ~~ationed~~ on BLI biosensors and  
355 for detection in AUC-FDS and MST, we ordered biotinylated and 5-Carbofluorescein (5-FAM)-  
356 labeled DNA oligonucleotides, respectively.

357 ~~To perform biophysical characterization measurements, samples of high purity, stability and~~  
358 ~~monodispersity are needed (Fig. S2).~~ All proteins were dialyzed to eliminate glycerol from the

359 storage buffer, which can interfere with the measurements. We chose the pH and ionic  
360 strength of the buffer for the best solubility of the samples. To simplify as much as possible,  
361 we used the dialysis buffer (20 mM Tris-HCl pH 8, 150 mM NaCl, 5 mM  $\beta$ -mercaptoethanol) as  
362 running buffer in most experiments. Due to the particularities of each approach and  
363 measurement devices, there are some limitation in the buffer choice. In some cases, blocking  
364 agents were required, such as 1mg/mL BSA, especially for low protein concentrations (in the  
365 nM range), to prevent surface adsorption. To reduce surface tension in capillaries and  
366 microfluidic devices, as well as detergents were required, such as 0.1 % Tween-20, ~~to reduce~~  
367 ~~surface tension (capillaries, chip, biosensors)~~. For AUC-FDS, we had to avoid Tris-HCl or HEPES  
368 above 20 mM, which can cause problems at 230 nm wavelength, and be aware of other  
369 absorbent molecules (nucleotides, old DTT or  $\beta$ -mercaptoethanol). For MST experiments, we  
370 used the commercial MST buffers (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 10 mM MgCl<sub>2</sub>, 0.05  
371 % Tween-20) for Ku-DNA and, Roti®-Stock 1x PBS (10 mM Na<sub>2</sub>HPO<sub>4</sub>, 2 mM KH<sub>2</sub>PO<sub>4</sub> pH 7.6137  
372 mM NaCl, 2.7 mM KCl, 0.005% Tween 20) for A3-Rep17. For switchSENSE sizing experiments  
373 D<sub>h</sub> estimation, a low salt buffer is required (10 mM Tris-HCl, 40 mM NaCl, 0.05 % Tween-20,  
374 50  $\mu$ M EGTA, 50  $\mu$ M EDTA).

375 In order for new users to determine the concentration ranges to work with in an unknown  
376 system, preliminary experiments need to be done using a broad range, observe the results  
377 and in consequence refine the initial conditions to a narrow range that could better suit the  
378 system in question. If an interaction partner is difficult to produce or not stable at high  
379 concentration, in solution approaches will be the one to keep in constant concentration during  
380 titration of its partner. In the other approaches must be the one to try first to immobilize or  
381 label. Reverse configuration can be always tested to improve or confirm the results.

## 382 Results

### 383 Biophysical measurements of alphaRep interactions

384 We characterized the interaction of A3 with its binders ~~Rep2 and~~ Rep17 (Rep2 was also used  
385 during the preparation of the training) using the following techniques presented in the Fig. 1:  
386 in solution approaches, which do not require labeling (SEC-MALS and ITC), in solution  
387 approaches that require labeled protein or DNA (MST and AUC-FDS), and surface approaches  
388 (BLI and switchSENSE). The students performed the measurements presented here during the  
389 training (Fig. 2, Fig. S23 and Table S1). ~~The studied interactions data represent limited value~~  
390 ~~due to students measuring and protein concentration as common denominators, but added~~  
391 ~~benefits of putting the techniques in context. For most of them, additional measurements and~~  
392 ~~controls are needed.~~ When available, we mention the values that have been reported for  
393 some approaches in previous studies/articles.

### 394 AlphaRep interactions measured by label free in solution approaches

395 Due to time limitations, the SEC-MALS experiments were performed ~~in our platform~~ prior to  
396 the training. We used a Superdex 200 increase 10/300 GL column (*Cytiva*), and compared  
397 three different runs with A3 alone, Rep2 alone and A3-Rep2 complex at 2 mg/mL  
398 ~~concentration~~ each. A3 eluted as a dimer with a molar mass of  $44.8 \pm 0.4$  kDa and, a  
399 hydrodynamic radius of  $3.0 \pm 0.2$  nm. Rep2 eluted as a monomer with a molar mass of  $11.6 \pm$   
400  $0.9$  kDa. The Rep2 concentration was insufficient, in view of the small molar mass, to make  
401 accurate sizing measurement by DLS. Finally, the A3-Rep2 complex eluted before the free  
402 proteins, with a molar mass of  $65.5 \pm 0.4$  kDa and hydrodynamic radius of  $4.0 \pm 0.2$  nm (Fig.  
403 2b). The stoichiometry was determined by using the protein conjugate method (Loiseau et al.,  
404 2017). Accurate mass can be calculated from MALS data with this method, which uses two  
405 concentration detectors simultaneously (a refractometer and UV spectrophotometer), and  
406 information about refractive index increment ( $dn/dc$ ) and UV extinction coefficient of each  
407 component. In our case, the complex corresponds to one dimer of A3 and two Rep2  
408 molecules.

409 Preparatory ITC experiments were performed ~~in our platform~~ on a PEAQ-ITC instrument with  
410 A3 in the cell at  $18 \mu\text{M}$  and Rep17 in the syringe at  $187 \mu\text{M}$  (Fig. S23a). Three different groups  
411 of students did a triplicate measurement (~~Fig. 2c~~) during the training using the same PEAQ-

412 ~~ITC~~ same instrument. We observed bi-phasic thermograms, and isotherms were fitted using a  
413 model of two identical binding sites with cooperativity. This cooperativity could stem from the  
414 propensity of A3 to form homodimers at  $\mu\text{M}$  concentration as shown by SEC-MALS (Freire et  
415 al., 2009; Vega et al., 2015). We could estimate two  $K_D$  values, the first  $K_D$  corresponds to an  
416 interaction with a tight affinity ( $K_{D1}$  ~~from 0.485~~ ~~to 0.667~~ nM) and the second one to a weaker  
417 affinity ( $K_{D2}$  ~~from 16~~ ~~to 32~~ nM) (Fig. 2c). With the obtained parameters for each site,  
418 described in the table at the bottom of thermograms, an average global affinity (Wyman and  
419 Gill, 1990) could be calculated (geometric mean of both dissociation constants:  $K_{D,av} = \sqrt{K_{D1}$   
420  $\times K_{D2}}$ ), which for each assay was 3.9 nM, 3.1 nM, and 3.2 nM, close to that obtained with other  
421 techniques. Data were analyzed during the training using the Origin software and the new  
422 PEAQ ITC software for comparison.

#### 423 **AlphaRep interactions measured by biophysical approaches with a partner immobilized on** 424 **a surface**

425 ~~Preliminary~~ ~~Comparable analysis using SPR classical technology, were carried out in our~~  
426 ~~platform using a ProteON XPR36 instrument from Biorad (data not published, Fig. S23b).~~

427 ~~Before the training p~~ Preliminary BLI experiments were performed ~~before the training~~ on an  
428 Octet RED384 instrument where His-tagged A3 protein was captured on Ni-NTA sensors at 5  
429  $\mu\text{g}/\text{mL}$  for 20 s and Rep17 concentration ~~ranges-ranged~~ from 200 nM to 1.56 nM. In these  
430 conditions, in which A3 is most probably in a monomeric form, the calculated kinetic rates  
431 were  $k_{ON}$  of  $1.3 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$ ,  $k_{OFF}$  of  $5.9 \times 10^{-3} \text{ s}^{-1}$  and  $K_D$  of 22.4 nM (data not shown). We observed  
432 that the fit was not correct with a single site model and that residuals showed systematic  
433 errors. During the training, a duplicate experiment was performed on an Octet RED96e in a  
434 buffer containing 1 mg/mL BSA and 0.1 % Tween-20 to limit the non-specific binding. In this  
435 case, the fitting was improved (Fig. 2d). We obtained the following preliminary values:  $k_{ON}$  of  
436  $2.6 \pm 0.7 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$ ,  $k_{OFF}$  of  $7.6 \pm 0.7 \times 10^{-4} \text{ s}^{-1}$ ,  $K_D$  of  $3.2 \pm 1.1 \text{ nM}$ . The  $K_D$  measured from the  
437 plateau of the curve (steady state) was of  $3.3 \pm 0.8 \text{ nM}$ , close to the one obtained from kinetics  
438 (Fig. S23c).

439 For the switchSENSE experiments, we first coupled A3 with the DNA strand (cNL-B48), which  
440 was complementary to the surface-tethered DNA strand on the chip. In this case, the surface-  
441 tethered DNA strand was labeled with a red fluorescent probe (NLB48-red dye). The DNA-



442 protein conjugate was purified using an anion-exchange-chromatography (Fig. S23d, top left).  
443 We then hybridized the A3-cNLB48 with the NLB48 on the sensor surface at a concentration  
444 of 100 nM. The hybridization step could be monitored in real-time by measuring the  
445 fluorescence increase (Fig. S23d, top right). Preliminary kinetic experiments were done using  
446 the dynamic mode (switching of the nanolevers) ~~in our platform~~. Rep2 alphaRep was used as  
447 analyte at three different concentrations ranging from 100 nM to 11.1 nM. Preliminary kinetic  
448 rates were calculated from a global fit for the three concentrations:  $k_{ON}$  of  $2.40 \pm 0.05 \cdot 10^{+6} \text{ M}^{-1} \text{ s}^{-1}$ ,  
449  $k_{OFF}$  of  $6.86 \pm 0.23 \cdot 10^{-4} \text{ s}^{-1}$  and  $K_D$  of  $0.29 \pm 0.01 \text{ nM}$  (Fig. S23d, bottom, left). For each  
450 analyte concentration, it was possible to perform a sizing experiment to measure the  
451 hydrodynamic diameter of the ligand A3, before or after association with Rep2. The  $D_h$  of the  
452 dimeric form of A3 calculated from the crystal structure (PDB 6FT) is found to be equal to 4.8  
453 nm. Due to protein dilution, we expect A3 to be present as a monomer on the DNA conjugated  
454 strand. Indeed, the  $D_h$  of  $3.0 \pm 0.1 \text{ nm}$  measured in our switchSENSE experiments is compatible  
455 with a monomeric form of A3 (Fig. S23d, bottom, right). The  $D_h = 3.8 \pm 0.1 \text{ nm}$  measured for  
456 the complex A3-Rep2 corresponds to an increase of 0.8 nm when compared to the  
457 hydrodynamic diameter of the monomeric form of A3 (Fig. S23d, bottom, left). During the  
458 training, kinetic experiments in static mode (nanolevers in up position) were performed, as  
459 before for Rep2, with Rep17 concentration ranging from 100 nM to 11.1 nM in the same  
460 conditions as before for Rep2, but with Rep17 alphaRep as ligand. The calculated kinetic values  
461 measured in this case were in the same range than the ones obtained previously with Rep2:  
462  $k_{ON}$  of  $2.35 \pm 0.17 \cdot 10^{+6} \text{ M}^{-1} \text{ s}^{-1}$ ,  $k_{OFF}$  of  $2.30 \pm 0.34 \cdot 10^{-3} \text{ s}^{-1}$  and  $K_D$  of  $0.98 \pm 0.16 \text{ nM}$  (Fig. 2e).  
463 During the training, students did not have time to run a sizing experiment.

464 ~~Comparable analysis using SPR classical technology, were carried out in our platform using a~~  
465 ~~ProteON XPR36 instrument from Biorad (data not published, Fig. S3b).~~

#### 466 **AlphaRep interactions measured by biophysical approaches with a labeled partner**

467 Because AUC-FDS experiment ~~were was~~ time consuming, data were collected before the  
468 training ~~in our platform~~. We first labeled A3 with the dye NT495 using the commercial  
469 *NanoTemper* Monolith Protein Labeling Kit BLUE-NHS (Amine Reactive). In these conditions,  
470 we were able to use a low concentration of A3 (5 nM). At this nanomolar concentration, A3 is  
471 a monomer as observed before by switchSENSE. We used variable concentrations of Rep17

472 from 0.5 nM to 0.5  $\mu$ M, and an An-50 Ti rotor at 42 000 rpm (130 000 g) speed. The  
473 sedimentation coefficients for A3 and A3-Rep17 complex were  $2.16 \pm 0.08$  S and  $2.96 \pm 0.05$   
474 S, respectively and the calculated  $K_D$  at equilibrium was  $13.4 \pm 1.8$  nM (Fig. 2fe).

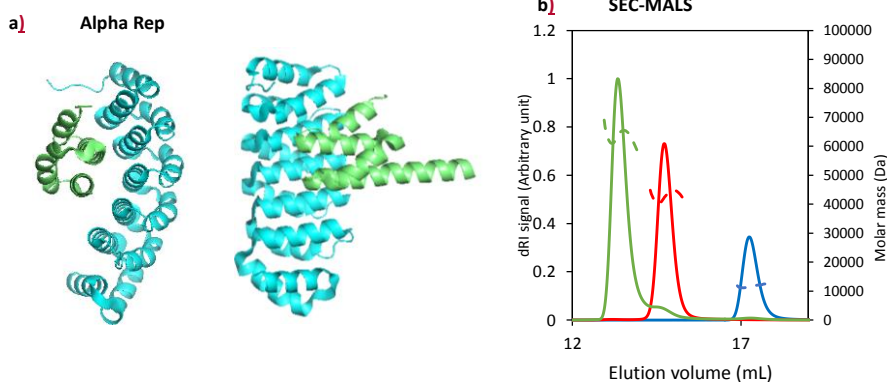
475 Finally, for the MST experiment, we decided to use ~~another the dye from NanoTemper~~  
476 ~~Monolith His-Tag Labeling Kit~~ Red-tris-NTA 2<sup>nd</sup> Generation ~~dye from the NanoTemper~~  
477 ~~Monolith His-Tag Labeling Kit~~, this site-specific non covalent labeling substantially improved  
478 the signal respect to the 1<sup>st</sup> Generation dyes, and respected better the integrity of protein.  
479 ~~Red-tris-NTA labeled A3 could not be used in AUC-FDS experiment, because its excitation and~~  
480 ~~emission maxima are out of the fluorescence detector range (488nm and 505-565 nm~~  
481 ~~respectively).~~ In ~~the~~ MST experiment, labeled A3 was at ~~70-35~~ nM, and the highest  
482 concentration of Rep17 was 8  $\mu$ M. The titration curves we obtained allowed us to measure a  
483  $K_D$  of  $8.0 \pm 4.3$  nM (Fig. 2g). ~~Using the labeled A3 produced for the AUC-FDS at 20 nM did not~~  
484 ~~provide us with substantial results due to high inhomogeneity in the sample preparation (Fig~~  
485 ~~S3e), thus leading to a strong noise and suggesting that this labeling of A3 influences the~~  
486 ~~interaction on the time scale of the MST measurement. Interestingly, we observed a titration~~  
487 ~~curve with a  $K_D$  at  $10.5 \pm 2.4$   $\mu$ M, which could be linked to an additional interaction between~~  
488 ~~A3-Rep17 complexes at the highest Rep17 concentration of 288  $\mu$ M or some steric hindrance~~  
489 ~~of the fluorescent probe. Orthogonal confirmation would help to better understand the origin~~  
490 ~~of this  $K_D$  difference, unfortunately none of the other approaches were performed at such~~  
491 ~~high concentration.~~

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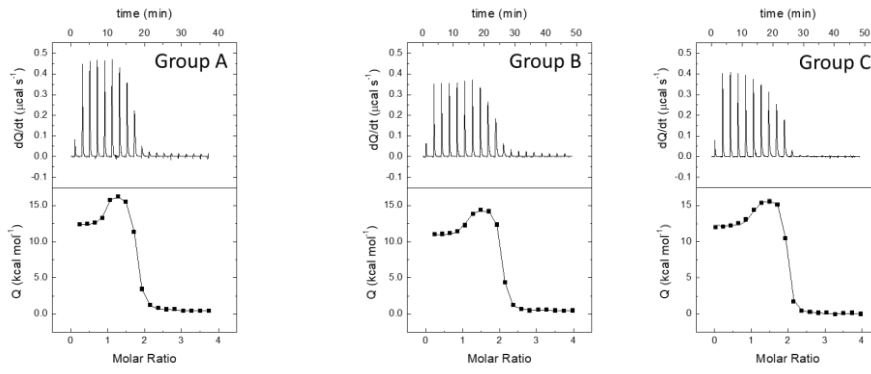
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492 **Figure 2. Biophysical measurements of alphaRep interactions.**



**c) ITC**

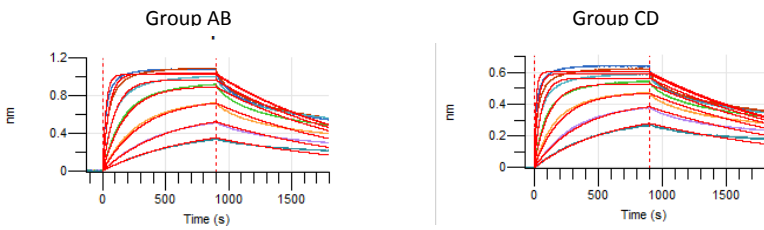


	K (M <sup>-2</sup> )	K <sub>0</sub> (nM)	ΔH (kcal/mol)	α	Δh (kcal/mol)	n
Group A	2.1 · 10 <sup>9</sup>	0.48	12.0	0.015	4.3	0.84
Group B	1.7 · 10 <sup>9</sup>	0.59	10.5	0.036	3.6	0.98
Group C	1.5 · 10 <sup>9</sup>	0.66	12.2	0.041	3.8	0.94

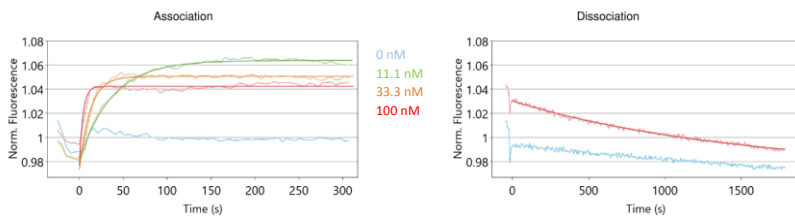
  

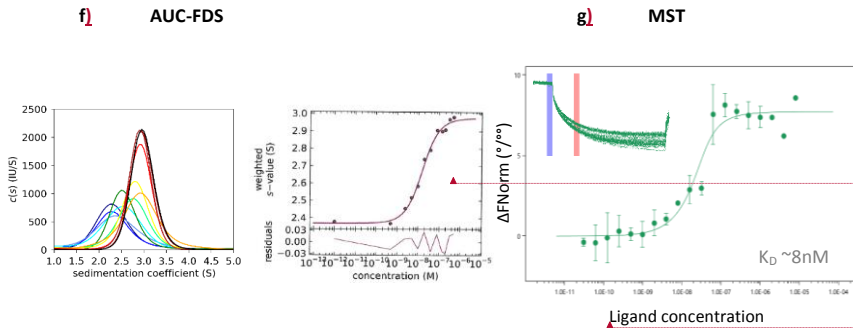
	K <sub>01</sub> (nM)	ΔH <sub>1</sub> (kcal/mol)	K <sub>02</sub> (nM)	ΔH <sub>2</sub> (kcal/mol)
Group A	0.48	12.0	32	16.3
Group B	0.59	10.5	16	14.1
Group C	0.66	12.2	16	16.0

**d) BLI**



**e) switchSENSE**





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493 a) Crystal structure of A3 alphaRep (a-A3, blue) in complex with Rep2 (bA3-2, green) (PDB: 4JW2) at 90° view  
 494 (left). b) SEC-MALS analysis. Elution profiles and molar masses of Rep2 in blue, A3 in red, and the complex of A3-  
 495 dimer and two molecules of Rep2 in green. c) PEAQ-ITC data of A3-Rep17 interaction obtained during the training  
 496 for three groups (A, B and C).  $K$ ,  $K_D$ , and  $\Delta H$  were the association constant, the dissociation constant, and the  
 497 interaction enthalpy of either of the two binding sites when the A3 dimer was unoccupied.  $\alpha$  and  $\Delta h$  were the  
 498 cooperative interaction constant and the cooperative interaction enthalpy, which reflect the binding cooperative  
 499 phenomenon (a factor that modulates the binding affinity and a term that modulates the binding enthalpy to the  
 500 second site when there was a site already occupied, respectively).  $n$  was the active (or binding-competent)  
 501 fraction of protein, since the stoichiometry was already included in the model.  $K_{D1}$  and  $\Delta H_1$  were the intrinsic  
 502 site-specific dissociation constant and binding enthalpy for the first binding site ( $K_{D1} = K_D, \Delta H_1 = \Delta H$ ).  $K_{D2}$  and  $\Delta H_2$   
 503 were the intrinsic site-specific dissociation constant and binding enthalpy for the second binding site ( $K_{D2} = K_{D1} /$   
 504  $\alpha$ ,  $\Delta H_2 = \Delta H_1 + \Delta h$ ). d) Octet RED96e BLI kinetic analysis of A3-Rep17 interaction in duplicate. Colors code of  
 505 Rep17 concentration: 3.13 nM (teal), 6.25 nM (purple), 12.5 nM (orange), 25 nM (green), 50 nM (cyan), 100 nM  
 506 (red), 200 nM (blue). e) switchSENSE kinetics analysis of A3-Rep17 interaction on dynamic mode, at three Rep17  
 507 concentrations. Normalized association (left) and dissociation (right) data were represented in function of time.  
 508 f) AUC-FDS distribution of sedimentation coefficients (left) with labeled A3 at 5 nM and increasing concentrations  
 509 of Rep17 from 0.5 nM to 0.5  $\mu$ M, obtained with the program SEDFIT (Schuck, 2000) and GUSSE (Brautigam, 2015).  
 510 Colors code of Rep17 concentrations: 0  $\mu$ M (navy blue), 0.5 nM (blue), 1 nM (cornflower blue), 2 nM (cyan), 4  
 511 nM (green), 8 nM (springgreen), 16 nM (yellow), 32 nM (orange), 64 nM (red), 128 nM (maroon), 256 nM (brown)  
 512 and 0.5  $\mu$ M (black). AUC-FDS  $K_D$  calculation (right), by fitting with isotherm tool in the program SEDPHAT. g) MST  
 513 analysis with red fluorophore. Inset, MST traces from relative fluorescence versus time, MST on used was 10 s  
 514 (red band).

515 In summary, the students obtained ~~preliminary results~~ in one week ~~with the six~~ using several  
 516 complementary approaches, results that were consistent in stoichiometry, and to a lesser  
 517 extent, in kinetic rates or affinity (Table S1). They could observe that the required ~~amount of~~  
 518 ~~material~~ quantity of sample, ligand or analyte, for each approach differs significantly. They also  
 519 observed that some approaches gave highly complementary information on the system  
 520 studied (Fig. S23). For example, ITC shows that the interaction is cooperative when A3  
 521 alphaRep is used at the high concentration needed for ITC (18  $\mu$ M). This cooperativity, linked  
 522 to the dimeric state of A3, was not observed with monomeric ligand immobilized on BLI or  
 523 switchSENSE. They observed higher  $K_D$  values with AUC-FDS and MST, where one of the  
 524 partner is labeled, reflecting possible steric hindrance between the fluorophore and the  
 525 interaction site. This was particularly true with the covalent amine labeling strategy where the  
 526  $K_D$  measured by MST was three order of magnitude higher.

527 **Biophysical measurements of Ku-DNA interactions**

528 We determined the interaction of the heterodimeric full-length protein Ku with four double-  
529 strand DNAs (dsDNAs) of different lengths: 18 bp, 42 bp, 48 bp, and 200 bp (Fig. 3, Fig. S34).  
530 Ku binds DNA through its ring-shaped structure (Fig. 3a). The main observations made during  
531 the training for the Ku-DNA interactions, ~~using the same six approaches, as for the alphaRep~~  
532 ~~interactions,~~ are shown in Fig. 3 and Table S2.

533 **Ku-DNA interactions measured by label-free in solution biophysical approaches**

534 For SEC-MALS, we used ~~a Superdex 200 increase 10/300 GL column (Cytiva) the same column~~  
535 as above and ran first a quadruplicate experiment with Ku alone, resulting in an average molar  
536 mass of 144.4 kDa (Fig. S34a). We then ran a duplicate experiment with Ku and a 1.2 excess of  
537 42 bp DNA. We obtained three peaks: the first corresponded to 2 Ku : 1 DNA complex (average  
538 Mw of 306.2 kDa), the second corresponded to 1 Ku : 1 DNA (average Mw of 171.0 kDa), and  
539 the last corresponded to the excess of dsDNA alone (average Mw of 26.1 kDa). This suggests  
540 that there is an equilibrium between 2:1 and 1:1 Ku-DNA complexes (Fig. 3b) with  
541 stoichiometries determined by using the protein conjugated method (Loiseau et al.; 2017).  
542 The presence of these two types of complexes could be explain by the use of a non-saturating  
543 Ku concentration.

544 For ITC experiments, we used in our laboratory two different sizes of dsDNA, 18 bp and 42 bp  
545 (Gontier ~~et al., 2021, Chapter of MiMB, in press~~). Due to time limitations students repeated  
546 the measurement only with the 42 bp. We used a VP-ITC instrument which consumes more  
547 sample, but was more sensitive to study these protein-DNA interactions. The heat effects were  
548 positive (endothermic interaction) and small ( $0.2 \mu\text{cal}\cdot\text{sec}^{-1}$ ) (Fig. 3c). Students did two runs,  
549 the first at 20  $\mu\text{M}$  and the second at 40  $\mu\text{M}$  concentration of DNA in the syringe, and in both  
550 cases Ku at 4  $\mu\text{M}$  in the cell. We obtained a mean  $K_D$  at equilibrium of  $3.7 \pm 0.7$  nM with a  
551 molar ratio of  $0.34 \pm 0.02$  and a  $\Delta H$  of  $24.4 \pm 2.7$  kcal/mol. The molar ratio was in good  
552 agreement with a ratio of 0.5 expected for the interaction of two Ku molecules with a DNA of  
553 42 bp. No evidence for cooperativity was observed. All the data were analyzed using the Origin  
554 software and the new PEAQ ITC software for comparison.

555 **Ku-DNA interactions measured by biophysical approaches with a partner immobilized on a**  
556 **surface**

557 Preparatory BLI experiments were performed on an Octet RED384 instrument. Firstly, we tried  
558 the ~~same~~ strategy that was used for the A3 alphaRep protein, i.e. ~~captured capturing the~~ Ku  
559 heterodimer on Ni-NTA sensors. We did not observe any binding with an 18 bp DNA as analyte  
560 (data not shown). We hypothesized that the DNA binding site may not be accessible when Ku  
561 is immobilized through its His-tag. We therefore tested another strategy, which relied on the  
562 capture of a biotinylated 42 bp DNA on Streptavidin sensors (at 10 nM for 120 s), which were  
563 then incubated with Ku protein as analyte (at concentrations in 200 nM to 1.56 nM range). In  
564 these conditions, we observed an interaction, with an estimated  $K_D$  around 40 nM (data not  
565 shown). We repeated the experiment in duplicate during the training on an Octet RED96e  
566 using an optimized buffer with 1mg/mL BSA and 0.1 % Tween-20, and Ku protein  
567 concentrations ranging from 200 nM to 3 nM. The results of the two runs were consistent,  
568 with apparent kinetic rates,  $k_{ON}$  of  $1.91 \pm 0.02 \cdot 10^{+6} \text{ M}^{-1}\text{s}^{-1}$ ,  $k_{OFF}$  of  $7.17 \pm 0.33 \cdot 10^{-4} \text{ s}^{-1}$ , and a  $K_D$   
569 of  $0.37 \pm 0.01 \text{ nM}$  (Fig. 3d). However, the deviation between the fitted curves and the  
570 experimental data was high, indicating that the interaction mechanism was more complex  
571 than a simple 1:1 binding. For instance, the association and the dissociation processes could  
572 be limited by the diffusion of Ku towards and from the biosensor surface (mass transport  
573 limitation). We therefore analyzed the concentration-dependence of the steady state  
574 responses and measured a  $K_D$  of  $5.2 \pm 0.2 \text{ nM}$  (Fig. S34b). As several curves did not reached a  
575 steady state, this  $K_D$  value could however be overestimated and experiments should be  
576 reproduced with longer association times. Taking everything into account, BLI binding  
577 constants were at this stage only qualitative.

578 Preliminary switchSENSE experiments were done before the training by forming an 80 bp  
579 dsDNA on the sensor. For that, we hybridized an 80-mer DNA with a 32-mer DNA. This DNA  
580 was complementary to the NL48 ssDNA on the chip (Fig. S34c, top, left). We performed kinetic  
581 analyses in duplicate using static mode and Ku protein at 500 nM concentration. We obtained  
582 the following kinetic rates:  $k_{ON}$  of  $9.3 \pm 0.1 \cdot 10^{+4} \text{ M}^{-1}\text{s}^{-1}$ ,  $k_{OFF}$  of  $2.8 \pm 0.1 \cdot 10^{-4} \text{ s}^{-1}$ , and  $K_D$  of  $3.1 \pm$   
583  $0.1 \text{ nM}$  (Fig. S34c, bottom). We monitored the dissociation over a long time (5000 s) to  
584 measure a significant proportion of dissociated Ku molecules. In between, we performed a  
585 sizing measurement. The calculated value ( $D_h = 8.9 \pm 0.3 \text{ nm}$ ) was in good agreement with the  
586 one obtained from the crystal structure of the Ku-DNA complex (PDB: 1JEQ;  $D_h = 8.9 \text{ nm}$ ) (Fig.  
587 S34c, top, right). During the training, we performed experiments in dynamic mode using a 48

588 bp DNA (without overhang). We used a Ku concentration range from 200 nM to 22.2 nM with  
589 1/3 serial dilution this time. In these conditions, the protein was closer to the fluorophore at  
590 the tip of the 48 bp DNA on the chip. We measured in these conditions a  $k_{ON}$  of  $2.6 \pm 0.2 \cdot 10^{+6}$   
591  $M^{-1}s^{-1}$ , a  $k_{OFF}$  of  $2.3 \pm 0.3 \cdot 10^{-3} s^{-1}$ , and a  $K_D$  of  $1.0 \pm 0.2$  nM (Fig. 3e). During the training, students  
592 did not have time to run a sizing experiment.

593 Comparable preliminary data were obtained by SPR ~~in our platform~~ using a ProteON XPR36  
594 instrument from *Biorad* (Fig. S34d, left), and ~~in Institut Pasteur platform using~~ a Biacore T200  
595 instrument from *Cytiva* (Fig. S34d, right).

#### 596 **Ku-DNA interactions measured by biophysical approaches with a labeled partner**

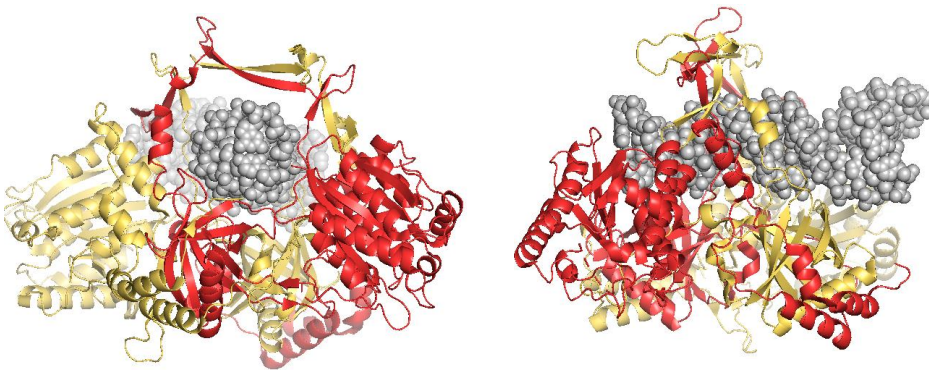
597 For AUC-FDS, we used an 18 bp DNA labeled with fluorescein (FAM) in 5'. We titrated the DNA  
598 (60nM) with Ku ~~protein from (concentration range from 0.5 nM to 0.5  $\mu$ M) concentrations~~  
599 ~~with 60nM of DNA~~. The sedimentation profile showed two species, corresponding to the DNA  
600 alone ( $2.15 \pm 0.05$  S) and to the 1:1 protein-DNA complex ( $7.25 \pm 0.15$  S), and we were able to  
601 determine the  $K_D$  at equilibrium ( $12.9 \pm 3.2$  nM) (Fig. 3f).

602 For MST, we used the same 18 bp labeled DNA ~~same labeled 18 bp DNA~~ as for AUC-FDS, but  
603 at 10 nM ~~concentration~~; and titrated it with Ku (concentration range from 2  $\mu$ M)  
604 concentration. Training participants performed four runs in total, in duplicates or triplicate  
605 ~~measurements~~ (Fig. 3g). We could see some variability in the curves that might originate from  
606 the pipetting of the different students. All curves were fitted globally resulting in a  $K_D$  of 2.8  
607 nM.

608 Comparable data were obtained in parallel of the training with a novel technology,  
609 microfluidic diffusional sizing (MDS) in collaboration with Fluidic Analytics (Fig. S34e). The  
610 Fluidity One-W instrument measures the rate of diffusion of macromolecules under steady  
611 state laminar flow in a microfluidic chip. In a diffusion chamber with two parallel streams, the  
612 migration of a labeled partner depends on its size. At the end, the streams are re-split and  
613 ~~from the fluorescence ratio of the fluorescence between the two allows to calculate~~,  $R_h$  ~~is~~  
614 ~~calculated~~. Then, ~~changes in average size after titrat~~ variation of  $R_h$  observed when titrating  
615 ~~an unlabeled binding partner against~~ the labeled partner by the unlabeled one allows to  
616 generate, give a binding curve and to calculate a  $K_D$  value.

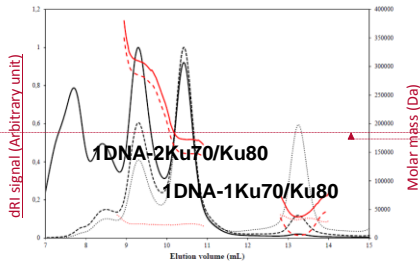
617 **Figure 3. Biophysical measurements of Ku-DNA interactions.**

a) Ku-DNA

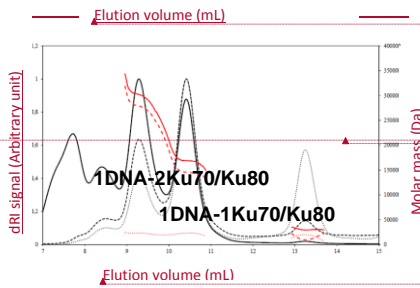




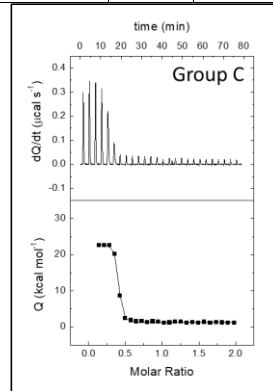
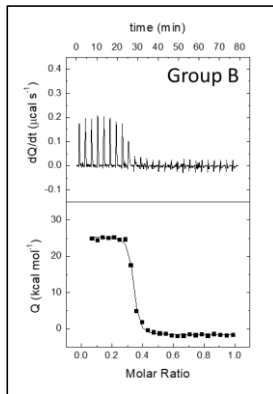
**b) SEC-MALS**



		Peak 1	Peak 2	Peak 3
Group C	Protein molar mass	279.1	145.7	9.6
	DNA molar mass	23.3	23.3	19.9
	Total molar mass	302.3	169.0	29.6
Group D	Protein molar mass	285.3	148.4	4.8
	DNA molar mass	24.7	24.6	32.3
	Total molar mass	310.1	172.9	37.1



**c) ITC**



	K (M <sup>-1</sup> )	K <sub>D</sub> (nM)	ΔH (kcal/mol)	n
Group B	3.4 10 <sup>8</sup>	3.0	27.1	0.32
Group C	2.3 10 <sup>9</sup>	4.3	21.7	0.37

**d) BLI: Group AB and Group CD**

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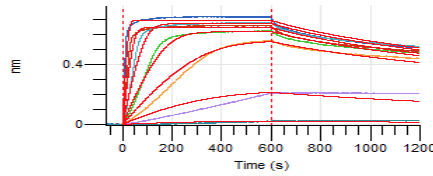
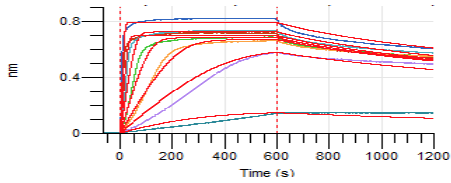
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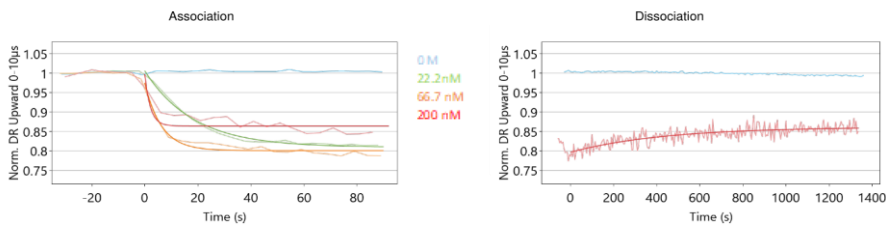
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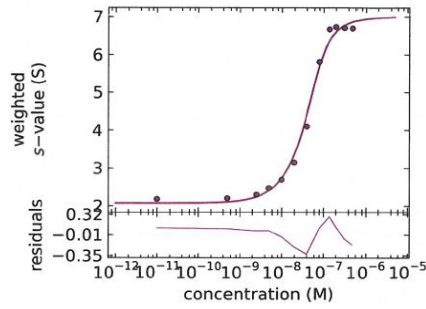
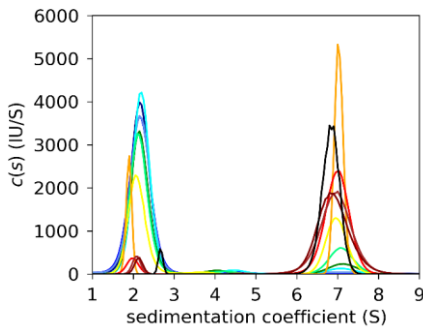
e) switchSENSE

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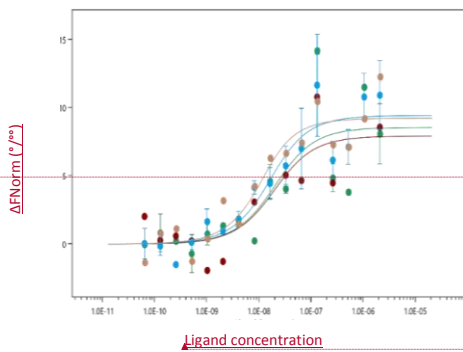
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f) AUC-FDS



g) MST



GROUPS	Excitation Power	$K_D$	$K_D$ Confidence	Std. Error of Regression	Signal to Noise
A1-B1	60	$1.53 \cdot 10^{08}$	$1.86 \cdot 10^{-08}$	2.77	3.33
A2	40	$1.49 \cdot 10^{08}$	$1.73 \cdot 10^{-08}$	2.01	4.33
B2-C2-D2	100	$1.14 \cdot 10^{08}$	$7.26 \cdot 10^{-09}$	1.61	6.31
D1	100	$6.15 \cdot 10^{09}$	$4.3 \cdot 10^{-09}$	1.56	6.37

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618 a) Crystal structure of the Ku- DNA complex (PDB: 1JEQ). View down the DNA helix (left) and side view (right),  
 619 with Ku70 colored in red, Ku80 in yellow cartoons and DNA in grey spheres. b) SEC-MALS elution profiles and  
 620 molar masses of Ku-DNA complex in duplicate. Representation of elution volumes in mL and molar mass in g/mol.

621 c) VP-ITC data in duplicate. d) Octet RED96e BLI kinetics data in duplicate. Colors code of Ku concentration: 3.13  
622 nM (teal), 6.25 nM (purple), 12.5 nM (orange), 25 nM (green), 50 nM (cyan), 100 nM (red), 200 nM (blue). e)  
623 switchSENSE dynamic mode data for three Ku concentrations. F) AUC-FDS measurement of Ku-DNA interaction.  
624 AUC-FDS distribution of sedimentation coefficients (left) with labeled DNA at 60 nM and increasing  
625 concentrations of Ku from 0.5 nM to 0.5  $\mu$ M, obtained with the program SEDFIT (Schuck, 2000) and GUSI  
626 (Brautigam, 2015). Colors code of Ku concentration: 0  $\mu$ M (navy blue), 0.5 nM (blue), 1 nM (cornflower blue), 2  
627 nM (cyan), 4 nM (green), 8 nM (springgreen), 16 nM (yellow), 32 nM (orange), 64 nM (red), 128 nM (maroon),  
628 256 nM (brown) and 0.5  $\mu$ M (black). AUC-FDS  $K_D$  calculation by fitting with isotherm tool in the program SEDPHAT  
629 (right). g) MST analysis. Representation of ligand concentration against  $\Delta F_{Norm} \text{ } ^{\circ}/^{\circ}$  (left). Superposition of  
630 several run in the similar conditions, but different excitation power and students. Summary of MST data (right).

631 In summary, ~~for the Ku-DNA project~~, the students also obtained ~~also for the Ku-DNA project~~  
632 ~~results~~ consistent ~~results of~~ stoichiometry, ~~and in the same range for~~ kinetic rates ~~or and~~  
633 affinity (Fig. 3, Table S2). As for the alphaRep project, ~~they observed that the amount of~~  
634 ~~material quantity of sample required~~ varies between the different approaches. ITC, SEC-MALS  
635 and AUC-FDS were very efficient for measuring the stoichiometry of Ku on DNA with one Ku  
636 molecule bound every 18-21bp DNA. They observed that measurements on surfaces, either  
637 by BLI or switchSENSE, were successful only when the DNA was immobilized. They could  
638 observe that switchSENSE allows a quite accurate estimation of the Ku size (Fig. S34d, bottom).  
639 Oligonucleotides with a fluorescent probe in 5' or 3' are inexpensive and allow to follow the  
640 interaction between a fluorescent DNA and a protein quite easily by MST or AUC-FDS, without  
641 major steric hindrance between the fluorescent probe and the interaction sites.

## 642 Discussion

643 The study presented here compares six different *in vitro* biophysical approaches to  
644 characterize the architecture (in terms of size or stoichiometry) and binding parameters ( $k_{ON}$ ,  
645  $k_{OFF}$ ,  $K_D$ ) of two different types of complexes (protein-protein and protein-DNA). It was  
646 designed to provide a rather complete overview of six different techniques in a short period.

647 During a week, 20 participants performed this study in the context of ~~a~~ the MoSBio Training  
648 School (ARBRE-MOBIEU COST Action). During the first day of the training school, experts of  
649 each field presented the projects, the theory and examples of applications for the three  
650 classical techniques (AUC, SEC-MALS, ITC), and the three more recent ones (MST, BLI,  
651 switchSENSE). An additional presentation about, sample quality control, a relevant subject  
652 regarding reproducibility of experimental measurements, completed the first day of the  
653 training (Raynal et al., 2014). The remainder of the week was dedicated fulltime to practical  
654 sessions where the participants could perform experiments on instruments, analyze results  
655 and discuss with experts. All six approaches, except AUC, and SEC-MALS for the alphaRep

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656 interaction, were successfully used during the training school to study two important types of  
657 macromolecular interactions (~~protein-protein~~alphaReps and ~~Ku~~protein-DNA). The training  
658 allowed the students, first, to compare the quantity of material consumed for each technique,  
659 and, second, to understand the parameters that can be measured by each of them (Fig. 4).  
660 This first edition of the MoSBio Training School was positively assessed ~~by~~ both ~~by~~ experts  
661 ~~trainers~~ and ~~participants~~trainees. It was a unique opportunity to compare advantages and  
662 limitations of this large ensemble of techniques. A new edition will be scheduled soon. The  
663 participants were able to use all instruments quite easily by themselves, with the exception of  
664 AUC, which requires a little more expertise and longer run times. Finally, this study showed  
665 that a nanomolar range affinity ~~is can be easily to be~~ assessed with all tested techniques. ~~The~~  
666 ~~students could observe that the  $K_D$  vary significantly with the technique used, though the  $K_D$~~   
667 ~~measured were all in the nanomolar range. The maximal and minimal values of  $K_D$  measured~~  
668 ~~between the alphaRep and the different techniques differ by a factor 21. The extreme  $K_D$  differ~~  
669 ~~by a factor 13 for the  $K_D$  measured between Ku and DNA. We observed that in these two~~  
670 ~~systems the presence of a label on one partner, in MST and AUC-FDS approaches, comes with~~  
671 ~~a higher  $K_D$  than without label. During the training school, the students could observe the~~  
672 ~~variability on the  $K_D$  values measured in the different groups or with the different approaches~~  
673 ~~(see Fig. 2 and Fig. 3). The training was also an opportunity to discuss on the techniques that~~  
674 ~~are more favorable Nevertheless, this is not the case when we want to measure lower  $K_D$ -(pM)~~  
675 or higher  ~~$K_D$ -( $\mu$ M)  $K_D$~~  values.

676 The results presented here for the alphaRep-proteins and Ku-DNA complexes highlight the  
677 advantages and drawbacks of each approach. The ~~quantity~~amount of required ~~material~~  
678 ~~sample~~ was not limited in our case, but the participants clearly observed that the amounts of  
679 protein and DNA used for each approach are very different (Fig. 4). AUC, SEC-MALS and ITC  
680 are the most sample-consuming techniques. ~~The~~ ITC200 and PEAQ-ITC ~~instruments~~ were an  
681 important progress for ITC in this regard, but we observed that for interactions with very weak  
682 heat exchange like Ku-DNA, the more sensitive, but more sample-consuming VP-ITC was still  
683 needed to obtain good results. Surface methods like BLI and switchSENSE require only small  
684 amounts of the immobilized protein. For the study of high affinity systems (in the nM range)  
685 like those characterized here, the consumption of analyte is small too. For weaker affinities  
686 (higher  $K_D$  in the  $\mu$ M range) the ~~required~~ ~~amount~~quantity of analyte will however rise

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687 significantly to cover concentrations from 1/10 of the  $K_D$  to 10 times the  $K_D$ . The MST technique  
688 consumes very small volumes of material once the labeling step is successfully achieved.  
689 Anyway, the time and sample consumption for a given technique must be evaluated not just  
690 for a single successful experiment, but must also ~~considering~~ take into account the  
691 experimental design and optimization stage.

692 **Figure 4. Comparison of results obtained for the alphaReps and the Ku-DNA interaction with**  
 693 **the six approaches.**

694

<b>Alpha Reps</b>	<b>SEC- MALS</b>	<u>200 µg A3, 200 µg Rep2</u>  Molar mass Stoichiometry: (A3-dimer): 2 Rep2	<b>ITC</b>	<u>80 µg A3, 130 µg Rep17</u>  Biphasic curve Cooperativity (A3 dimer) $K_{D1}, K_{D2}, \Delta H, -T\Delta S$	
	<b>BLI</b>	<u>8 µg A3, 2 µg Rep17</u>  A3-monomer $K_D, K_{ON}, K_{OFF}$	<b>Switch- SENSE</b>	<u>150 ng A3, 2-20 µg Rep17</u>  A3-monomer Size ( $D_b$ ) $K_D, K_{ON}, K_{OFF}$	
	<b>AUC- FDS</b>	<u>1 µg A3, 8 µg Rep17</u>  Sedimentation coefficient Stoichiometry $K_D$	<b>MST</b>	<u>620 ng A3, 6 µg Rep17</u> (One labeling failed)  $K_D$	
<b>Ku- DNA</b>	<b>SEC- MALS</b>	<u>40µg of DNA, 200 µg Ku</u>  Molar mass Stoichiometry: 1 Ku /18-21 bp	<b>ITC</b>	<u>990 µg Ku, 320 µg DNA</u>  Biphasic curve Stoichiometry: 1 Ku /18-21bp $K_D, \Delta H, -T\Delta S$	
	<b>BLI</b>	<u>400 ng DNA, 14 µg Ku</u> (Failed with Ku immobilized)  $K_D, K_{ON}, K_{OFF}$	<b>Switch- SENSE</b>	<u>150 ng DNA, 2-20 µg Ku</u> (Ligand is the nanolever)  Size ( $D_b$ ) $K_D, K_{ON}, K_{OFF}$	
	<b>AUC- FDS</b>	<u>3.5 µg DNA, 80 µg Ku</u> (DNA easily labeled)  Sedimentation coefficient Stoichiometry $K_D$	<b>MST</b>	<u>50 µg DNA, 12 µg Ku</u> (DNA easily labeled)  $K_D$	
<b>SEC- MALS</b>	<b>AlphaReps</b> ✓ 200 µg A3, 200 µg Rep2 ➤ Molar mass ➤ Stoichiometry: (A3 dimer): 2 Rep2		<b>ITC</b>	<b>AlphaReps</b> ✓ 80 µg A3, 130 µg Rep17 ✓ Biphasic curve ➤ Cooperativity (A3 dimer) ➤ $K_{D1}, K_{D2}, \Delta H, -T\Delta S$	
	<b>Ku-DNA</b> ✓ 40µg of DNA, 200 µg Ku ➤ Molar mass ➤ Stoichiometry: 1 Ku /18-21 bp			<b>Ku-DNA</b> ✓ 990 µg Ku, 320 µg DNA ✓ Biphasic curve ➤ Stoichiometry: 1 Ku /18-21bp ➤ $K_D, \Delta H, -T\Delta S$	
<b>BLI</b>	<b>AlphaReps</b> ✓ 8 µg A3, 2 µg Rep17 ✓ A3 monomer ➤ $K_D, K_{ON}, K_{OFF}$		<b>switchSENSE</b>	<b>AlphaReps</b> ✓ 150 ng A3, 2-20 µg Rep17 ✓ A3 monomer ✓ Size ( $D_b$ )	

			✓ $K_D, k_{ON}, k_{OFF}$
	<b>Ku-DNA</b> ✓ 400 ng DNA, 14 $\mu$ g Ku ✓ Failed with Ku immobilized ➤ $K_D, k_{ON}, k_{OFF}$		<b>Ku-DNA</b> ✓ 150 ng DNA, 2-20 $\mu$ g Ku ✓ Ligand is the nanolever ✓ Size ( $D_w$ ) ➤ $K_D, k_{ON}, k_{OFF}$
<b>AUC-FDS</b>	<b>AlphaReps</b> ✓ 1 $\mu$ g A3, 8 $\mu$ g Rep17 ➤ Sedimentation coefficient ➤ Stoichiometry ➤ $K_D$	<b>MST</b>	<b>AlphaReps</b> ✓ 620 ng A3, 6 $\mu$ g Rep17 ✓ One labeling failed ➤ $K_D$
	<b>Ku-DNA</b> ✓ 3.5 $\mu$ g DNA, 80 $\mu$ g Ku ✓ DNA easily labeled ➤ Sedimentation coefficient ➤ Stoichiometry ➤ $K_D$		<b>Ku-DNA</b> ✓ 50 $\mu$ g DNA, 12 $\mu$ g Ku ✓ DNA easily labeled ➤ $K_D$

695 The experiments performed by the students on the two systems of study with the six approaches were  
696 successful. ~~Quantity of materials~~The sample quantities used for the different approaches are indicate, as well as  
697 very different. Here are the amount needed per run. The training highlighted the parameters that can be deduced  
698 from the different approaches.

699 All experimental approaches used in this study can provide  $K_D$  values of the interaction.  
700 Interestingly, they also provide additional information that may orient the users to one or the  
701 other according to the main questions raised in their specific projects. In the two examples we  
702 studied, the stoichiometry issue is an important point, since the A3 alphaRep is a dimer when  
703 its concentration is in the  $\mu$ M range, while it is a monomer in the nM range. According to this  
704 oligomeric state, we can observe some cooperative binding of its partner ~~or not, or some~~which  
705 influences ~~on~~ the binding parameters. In this regards, ITC, SEC-MALS and AUC allow  
706 characterizing the ratio between the dimeric alphaRep target and its binders or between Ku  
707 and DNA of increasing lengths, and confirm the information provided by the ~~if~~ crystal  
708 structures of the complexes. The sizing measurement in switchSENSE can also provide some  
709 information regarding the stoichiometry or conformation.

710 Kinetic parameters are important data to estimate the half-time of a complex, which is an  
711 important interaction parameter ~~gaining attention recently~~. BLI and switchSENSE (as well as  
712 SPR) proved to be efficient in this study to determine  $k_{ON}$  and  $k_{OFF}$ . We observed that the  
713 switchSENSE allows to characterize very tight interactions with small  $k_{OFF}$  values ( $k_{OFF}$  about  
714  $10^{-4}$  s) by monitoring the dissociation over a long time period (5000 s). Thermodynamic  
715 parameters (enthalpy, entropy and Gibbs free energy) also provide key information about

716 macromolecular interactions, notably about the polar/apolar nature of the interface between  
717 a protein and its ligand. The Ku-DNA thermograms for example indicate that the interaction is  
718 entropically driven with a positive and unfavorable enthalpy probably linked to desolvation of  
719 the DNA and Ku surface upon binding. ~~The MoSBio training school over a week with 20~~  
720 ~~students proved to be a very rich occasion for such discussions.~~

721 The choice of one technique over another depends mainly on the questions one wants to  
722 answer. Some practical issues can also guide the choice of the method, such as the quantity  
723 and solubility of protein and ligands available, the possibility to immobilize or to label one of  
724 the interaction partners, and the time available. The presence of an instrument and experts  
725 in house or nearby the user is another criterion. Noteworthy, all these approaches are  
726 available in many research institutions in Europe, especially in those engaged in Structural  
727 Biology studies. If needed, these approaches are also available through network-research  
728 infrastructures, like MOSBRI and Instruct in Europe or FRISBI in France. Finally, there are  
729 research networks, like ARBRE-MOBIEU, allowing and-to fostering fruitful exchanges with  
730 experts in the different fields. The MoSBio training school over a week with 20 students proved  
731 to be a very rich occasion for such discussions.

732 Studying macromolecular interactions *in vitro* ~~are-is~~ extremely complementary to *in cellulo*  
733 analyses. Interactions observed in cells or in cellular extracts ~~need~~ often need further *in vitro*  
734 ~~validation-characterization and-of~~ target engagement ~~to confirm the specificity of the~~  
735 ~~proposed interaction~~. Training schools, as the one described in this article, are central to  
736 further disseminate the importance of biophysical studies in cellular biology laboratories. This  
737 training school contributed to initiate students from various fields ~~on-to~~ biophysical  
738 approaches and showed in a short period the added-value of quantitative measurements of  
739 protein interactions.

740 By mutagenesis, Nuclear Magnetic Resonance (NMR) shift or Hydrogen-Deuterium Exchange-  
741 Mass Spectrometry (HDX-MS), one can determine the residues involved in the interaction, and  
742 structural analysis can yield information about their topology. One can play with the  
743 experimental conditions (pH, ionic strength, temperature) to expand the knowledge about the  
744 nature of the interaction under study (electrostatic and/or hydrophobic, exo- or endothermic,  
745 presence/absence of conformational changes, coupling with additional binding equilibria).  
746 Furthermore, pharmacological studies require this kind of biophysical approaches. Finally,



747 molecular scale biophysics data can also be ~~also~~ used to implement *in silico* simulations to  
748 predict other interactions in basic research. A second edition of this Training School will be  
749 held as soon as possible. The extremely positive feedbacks from the students, as well as ~~and~~  
750 from the academic experts and industrials participating in this school, highlights the need for  
751 a better understanding of the theoretical bases ~~and hands-on experimental practical~~ of such  
752 a panel of biophysical methods coupled to hands-on experimental practice.

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847 **Declarations**

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851 **Conflicts of interest/Competing interests** The authors declare no competing interest. Pierre  
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853 commercial interest

854 **Ethics approval** Not applicable.

855 **Consent to participate** The authors consent to participate to this project.

856 **Consent for publication** The authors consent to publish the work reported in this paper.

857 **Availability of data and material** Data can be obtained by requesting the corresponding  
858 author.

859 **Code availability** Not applicable.

860 **Authors' contributions** PFV, PE, SU, CE, AVC, AR, JBC authors contributed to the study  
861 conception and design. Material preparation, data collection and analysis were performed by  
862 CV, MAN, SU, PE, AVC, DS, GB, PS, CQ, CE, AR. The first draft of the manuscript was written by  
863 PFV and all authors commented on previous versions of the manuscript. All authors read and  
864 approved the final manuscript.

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877 (<https://www.pluginlabs-universiteparisclay.fr/fr/results/keywords/PIM>), while some  
878 others were performed in Institut Pasteur, PFBMI platform.

879 **Supplementary material**

880 **Figures**

881 **Figure S1. Flyer of the Molecular Scale Biophysics (MosBio):**

Gif-sur-Yvette, France

June 3<sup>rd</sup> - 7<sup>th</sup> 2019

ARBRE-MOBIEU Training School on  
**Macromolecular Interactions**  
comparing *in vitro* methodologies

ITC  
Isothermal Titration Calorimetry

SEC/MALS  
Size Exclusion Chromatography  
Multi-Angle Light Scattering

AUC  
Analytical UltraCentrifugation

From classical to innovative approaches:  
Principles and Practice

switchSENSE®  
Molecular Dynamics Measurement

MST  
MicroScale Thermophoresis

BLI  
Bio-Layer Interferometry

**IZBC organisers:** CHARBONNIER Jean-Baptiste, FERNANDEZ-VARELA Paloma,  
MENETREY Julie, MINARD Philippe, NICAISE-AUMONT Magali & VELOURS Christophe

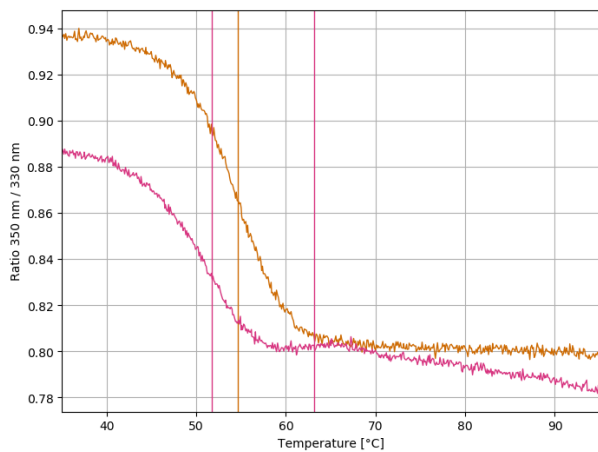
**External Speakers and Trainers:**  
BARON Bruno, BRULE Sébastien, ENGLAND Patrick & RAYNAL Bertrand, (Institut Pasteur, Paris, France)  
BEC Guillaume & ENNIFAR Eric (IBMC, Strasbourg, France)  
EBEL Christine (IBS, Grenoble, France)  
ROUSSEL Alain (AFMB, Marseille, France)  
STRICK Terence & STROEBEL David (ENS, Paris, France)  
UEBEL Stephan (MPI Biochemistry, Martinsried, Germany)  
VELAZQUEZ CAMPOY Adrian (BIFI, Zaragoza, Spain)

<https://mosbio.sciencesconf.org/>

882 **Macromolecular interactions *in vitro*, comparing classical and innovative approaches training school.** Students  
883 **during this training were able to use different approaches: Hydrodynamic (AUC-FDS, MST, SEC-MALS); Real-time**  
884 **biosensor (BLI, switchSENSE) and Thermodynamic (ITC) to measure *in vitro* macromolecular interactions (protein-**  
885 **protein and protein-DNA). Experts on these approaches came from France and Europe as speakers and trainers.**  
886 **This event was made possible thanks to several networks and sponsors.**



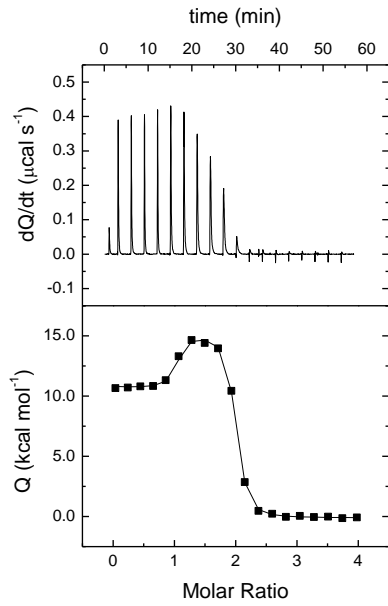
887 **Figure S2S1.** Unfolding profile plot of Ku70/Ku80.



888 Quality and stability of samples are crucial to obtain accurate biophysical data. For all the biophysical studies  
889 shown in this manuscript, we used a full-length version of Ku (called KuFL), but for structural studies we used a  
890 shorter version where the C-terminus of each monomer is deleted (called KuCC). We took advantage of the new  
891 technology of *NanoTemper*, the Tycho NT.6 instrument, present during the training, to compare the thermal  
892 stability of these two versions of Ku protein by a fast measurement. Fluorescence intensity is recorded at 330 nm  
893 and 350 nm (emission profile of the Tryptophan residues). The brightness ratio 350 nm / 330 nm plotted against  
894 the temperature is called the unfolding profile plot and inflexion temperatures can be derived representing  
895 unfolding events. Tryptophan fluorescence of KuFL (orange) and KuCC (pink) were follow during a ramp of  
896 temperature of 35-95°C. KuFL showed a higher temperature of unfolding than KuCC (vertical bars). Thus, KuFL  
897 appears to be more stable than KuCC by a few degrees.

898 **Figure S23.** Additional biophysical measurements of alphaRep interactions.

a) ITC



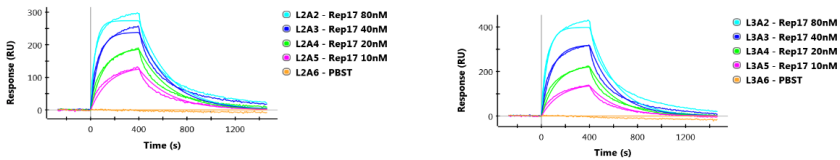
K (M <sup>-1</sup> )	K <sub>D</sub> (nM)	ΔH (kcal/mol)	α	Δh (kcal/mol)	n
1.4 · 10 <sup>9</sup>	0.71	10.9	0.024	4.4	0.95

K (M <sup>-1</sup> )	K <sub>D</sub> (nM)	ΔH (kcal/mol)	α	Δh (kcal/mol)	n
1.4 · 10 <sup>9</sup>	0.71	10.9	0.024	4.4	0.95

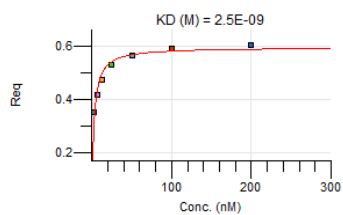
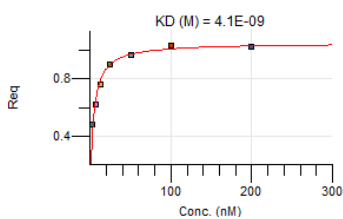
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b) SPR

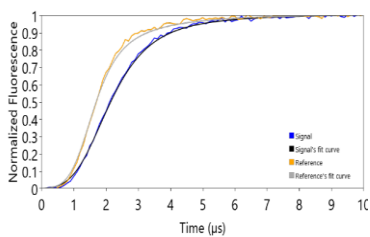
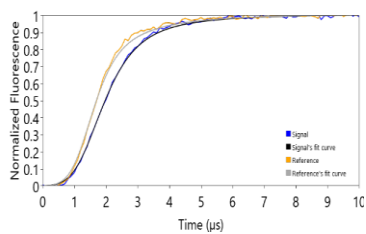
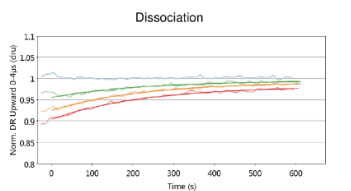
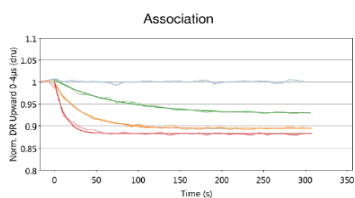
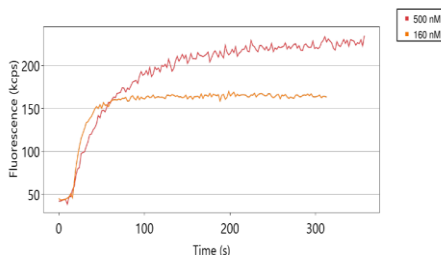
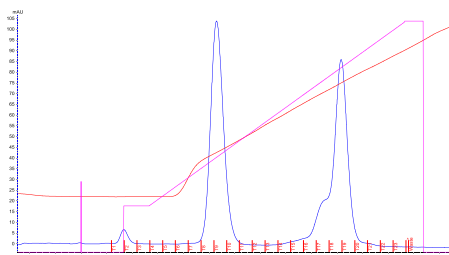




c) BLI



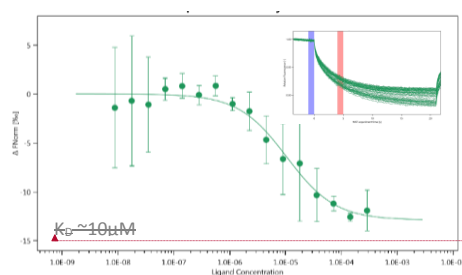
d) switchSENSE



$D_h^{298} = 3.02 \pm 0.05 \text{ nm} (n=8)$

$D_h^{298} = 3.82 \pm 0.09 \text{ nm} (n=4)$

e — MST



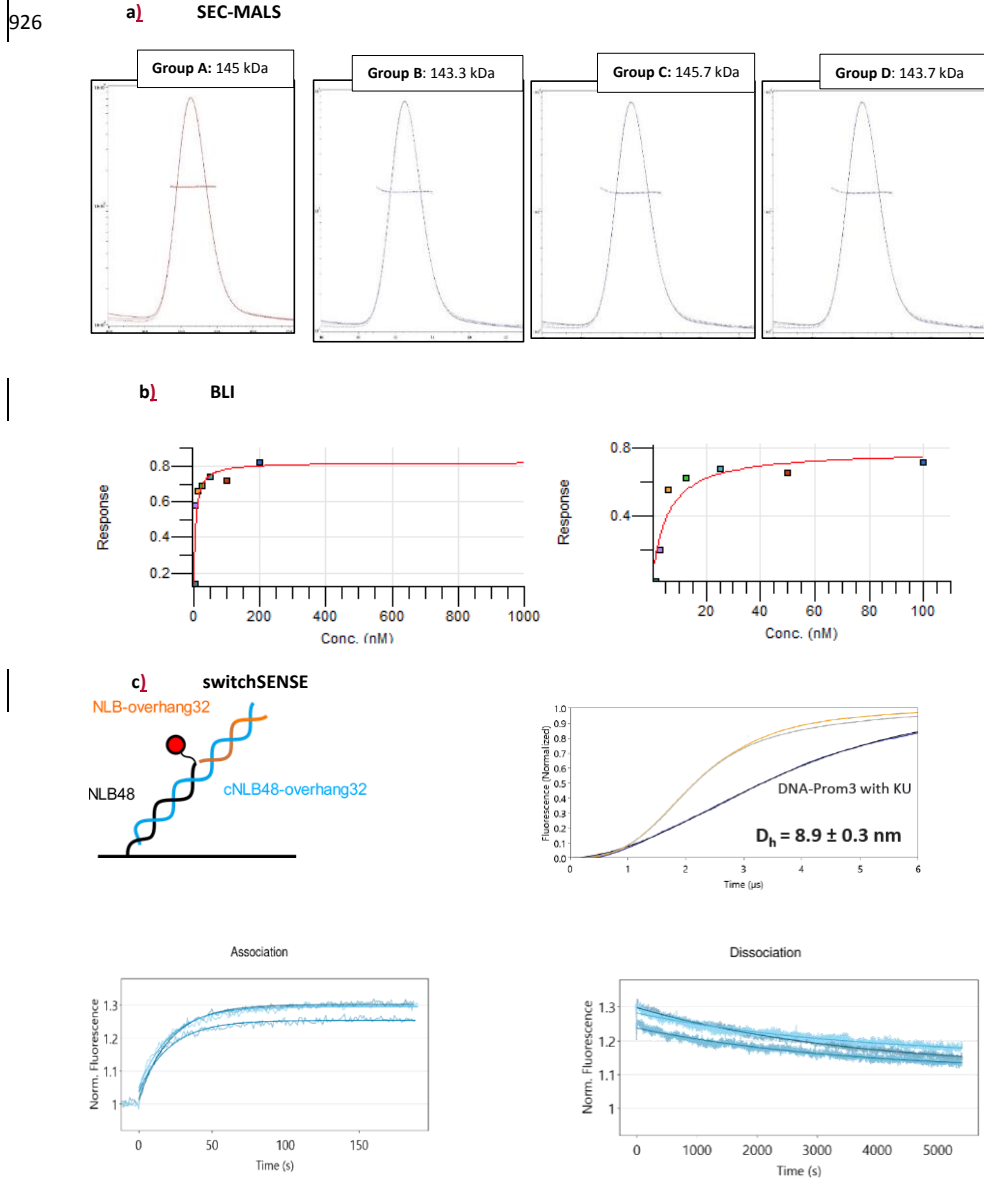
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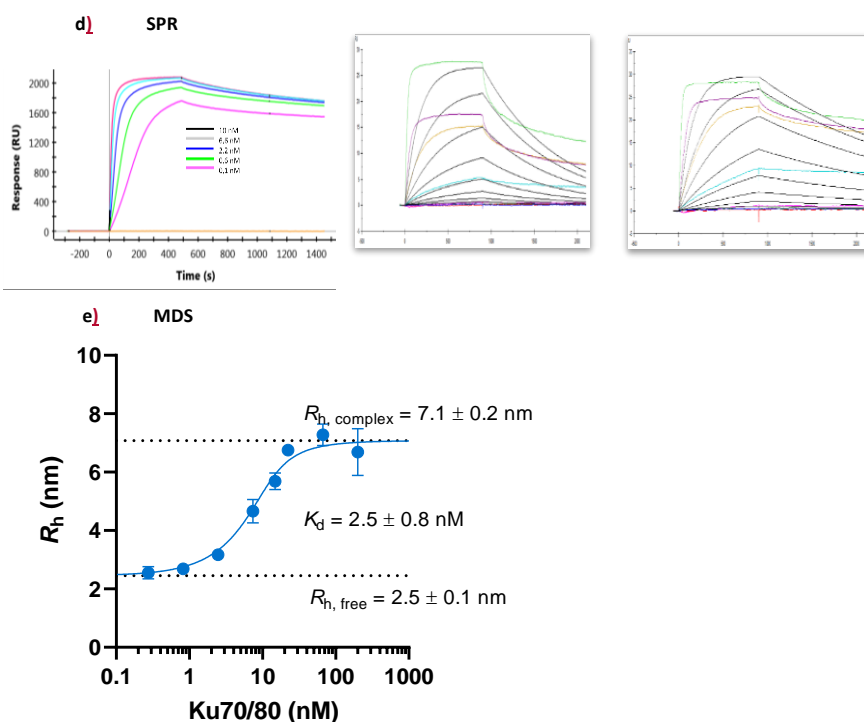
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899 a) PEAQ-ITC data obtained before the training in our laboratory.  $K$ ,  $K_D$ , and  $\Delta H$  were the association constant, the  
900 dissociation constant, and the interaction enthalpy of either of the two binding sites when the dimer was  
901 unoccupied.  $\alpha$  and  $\Delta h$  were the cooperative interaction constant and the cooperative interaction enthalpy, which  
902 modulate the binding cooperative phenomenon (a factor that modulates the binding affinity and a term that  
903 modulates the binding enthalpy to the second site when there was a site already occupied, respectively).  $n$  was  
904 the active (or binding-competent) fraction of protein, since the stoichiometry was already included in the model.  
905  $K_{D1}$  and  $\Delta H_1$  were the intrinsic site-specific dissociation constant and binding enthalpy for the first binding site  
906 ( $K_{D1} = K_D$ ,  $\Delta H_1 = \Delta H$ ).  $K_{D2}$  and  $\Delta H_2$  were the intrinsic site-specific dissociation constant and binding enthalpy for  
907 the second binding site ( $K_{D2} = K_{D1} / \alpha$ ,  $\Delta H_2 = \Delta H_1 + \Delta h$ ). b) Sensograms of alphaRep A3-Rep17 interaction measured  
908 in duplicate on a ProteON XPR36 instrument from *Biorad*. For this experiment, association and dissociation times  
909 were 400 s and 1000 s, respectively with a flow rate of 50  $\mu\text{L}/\text{min}$  in phosphate buffered saline with 0.1 % Tween-  
910 20 (PBST) using His-Tag capturing (HTG) chip to immobilized A3 at 6.25 or 12.5  $\mu\text{g}/\text{mL}$  during 80 s (inducing 60  
911 RU and 95 RU, respectively). Rep17 ranges from 10 to 80 nM concentration. A  $k_{\text{ON}}$  of  $2.8 \cdot 10^{+5} \text{ M}^{-1}\text{s}^{-1}$ , a  $k_{\text{OFF}}$  of  $3.9$   
912  $10^{-3} \text{ s}^{-1}$  and a  $K_D$  of 13.8 nM were measured in the first run, and a  $k_{\text{ON}}$  of  $1.8 \cdot 10^{+5} \text{ M}^{-1}\text{s}^{-1}$ , a  $k_{\text{OFF}}$  of  $4.6 \cdot 10^{-3} \text{ s}^{-1}$  and a  
913  $K_D$  of 25.9 nM in the second one. c) Octet RED96e BLI steady-state analysis in duplicate. Colors code of Rep17  
914 concentrations: 3.13 nM (teal), 6.25 nM (purple), 12.5 nM (orange), 25 nM (green), 50 nM (cyan), 100 nM (red),  
915 200 nM (blue). **d)** Sample preparation for switchSENSE experiments. Anion-exchange chromatogram of the A3  
916 cross-linked protein (up, left). The cross-linked protein was the shoulder of the final peak that correspond to the  
917 free DNA. Hybridization of the cross-linked ssDNA-A3 on the chip, which carries a red fluorescent probe (up,  
918 right). Red profile corresponds to the control (free cNLB48 without protein) and the orange one to the cross-  
919 linked cNLB48-A3 conjugate. switchSENSE dynamic mode data of A3-Rep2 at three concentrations (middle, left).  
920 Normalized association (left) and dissociation (right) data were represented in function of time. switchSENSE  
921 sizing measurement of A3 hydrodynamic diameter ( $D_h$ ) (bottom, left), and of the A3-Rep17 complex (bottom,  
922 right). Measured signal curve is in blue (conjugated DNA), the associated fitted curve in blank, reference signal  
923 (cNLB48) is in orange, and the associated fitted curve in grey. **d)** MST analysis with blue fluorophore. Inset, MST  
924 traces, MST on used was 10 s (red band).

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925 **Figure S34. Additional biophysical measurements of Ku-DNA interactions.**





927 a) SEC-MALS elution profiles and molar masses of Ku alone in quadruple. b) Octet RED96e BLI steady-state data  
 928 in duplicate. Colors code of Ku concentration: 3.13 nM (teal), 6.25 nM (purple), 12.5 nM (orange), 25 nM (green),  
 929 50 nM (cyan), 100 nM (red), 200 nM (blue). c) switchSENSE measurement set up (top, left). By hybridization of  
 930 complementary DNA carrying the target sequence as overhang, the surface is functionalized with the sequence  
 931 of interest. switchSENSE static mode data of 1 Ku concentration in duplicate (bottom). switchSENSE sizing data  
 932 (top, right). Reference (bare DNA) is depicted in yellow. DNA-protein-complex is depicted in blue. d) SPR  
 933 sensograms by ProteON XPR36 of Ku-200 bp dsDNA interaction measured (right). A 200 bp biotinylated DNA was  
 934 immobilized on a Streptavidin (NLC) chip. Ku70/Ku80 ranged from 0.1 to 10 nM concentrations, one heterodimer  
 935 may induce 200 RU. A  $k_{ON}$  of  $7.8 \pm 4 \cdot 10^{+6} \text{ M}^{-1}\text{s}^{-1}$ , a  $k_{OFF}$  of  $1.5 \cdot 10^{-4} \text{ s}^{-1}$  and a  $K_D$  of  $7.3 \pm 3 \text{ nM}$  were measured. This  
 936 experiment showed the threading of Ku on DNA, approximately 10 heterodimers bound to this 200 bp DNA. SPR  
 937 sensograms by BIACore T200 of Ku-42 bp dsDNA (middle) and, Ku-60 bp dsDNA (left) interaction measured by  
 938 biotinylated DNA immobilized in a serie S sensor chip SA at 10 nM concentration. Same buffer as in switchSENSE  
 939 experiment adding 0.2 mg/mL of BSA, flow rates of 20 to 100  $\mu\text{L}/\text{min}$ , and Ku at 5 nM as higher concentration for  
 940 900 s of association and dissociation. A  $k_{ON}$  of  $1.0 \cdot 10^{+6} \text{ M}^{-1}\text{s}^{-1}$ , a  $k_{OFF}$  of  $11.7 \cdot 10^{-4} \text{ s}^{-1}$  and a  $K_D$  of 1.2 nM ( $R_{\text{max}}$  of 32.9  
 941 RU) were measured for the 42 bp DNA and, a  $k_{ON}$  of  $1.4 \cdot 10^{+6} \text{ M}^{-1}\text{s}^{-1}$ , a  $k_{OFF}$  of  $5.0 \cdot 10^{-4} \text{ s}^{-1}$  and a  $K_D$  of 35.4 nM ( $R_{\text{max}}$   
 942 of 31.6 RU) for the 60 bp DNA. Colors code of Ku concentrations: 5 nM (light green), 2.5 nM (purple), 1.25 nM  
 943 (orange), 0.625 nM (cyan), 0.3125 (pink), 0.156 (dark green), 0.078 (blue), 0.039 (red). The differences observed  
 944 in the results depend mainly on the setup strategy of the experiment. Nevertheless, open questions remain to  
 945 answers: the purity of dsDNA and the stability of the protein will affect the results, but there is also the possibility  
 946 of different ways of DNA fixation by Ku. e) In collaboration with *Fluidic Analytics*, we collected preliminary data  
 947 to test their new instrument, Fluidity One-W with the Ku-DNA interaction. This is a novel technique in solution  
 948 based in diffusional sizing of a complex in a microfluidic system. For this experiment we used the ~~same~~-18 bp  
 949 dsDNA-FAM as before (AUC, MST) at 10 nM concentration, and 1/3 dilution of Ku70/Ku80 from 200 to 0.09 nM  
 950 concentration. We were able to measure  $R_h$  (DNA alone, complex) and  $K_D$  values.

951 Tables

952 Table S1. Summary of the results for the interaction between alphaRep proteins  
 953 interaction using different techniques.

	AUC-FDS	SEC-MALS*	ITC		MST	switchSENSE		BLI		SPR*
His-A3	Blue-NHS	Dimer	Monomer /high affinity	Dimer /low affinity	Red-His	Rep2*	Rep17	Monomer		Monomer
Ratio	1:1	1:1	1:1	1:1	N/A	1:1	1:1	N/A		N/A
K <sub>o</sub> (nM)	13.4 ± 1.8	N/A	0.6 ± 0.1	21.3 ± 7.5	8.0	0.29 ± 0.01	0.98 ± 0.16	3.2 ± 1.1	3.3 ± 0.8	3.97 ± 1.38
K <sub>ON</sub> (M <sup>-1</sup> s <sup>-1</sup> ) K <sub>OFF</sub> (s <sup>-1</sup> )	N/A	N/A	N/A	N/A	N/A	2.4 ± 0.1 10 <sup>-6</sup> 6.9 ± 0.2 10 <sup>-4</sup>	2.4 ± 0.2 10 <sup>-6</sup> 2.3 ± 0.3 10 <sup>-3</sup>	2.6 ± 0.7 10 <sup>-5</sup> 7.6 ± 0.7 10 <sup>-4</sup>	Steady-state	2.31 ± 1.79 10 <sup>-5</sup> 4.28 ± 0.36 10 <sup>-3</sup>
Other information	S (A3) = 2.6 ± 0.1 S (A3/Rep17) = 3.0 ± 0.1	Mw (Rep2) = 11.6 kDa Mw (A3) = 44.8 kDa R <sub>h</sub> (A3) dimer = 3nm Mw (A3/Rep2) = 65.5 kDa R <sub>h</sub> (A3/Rep2) = 3.8 nm	ΔH = 11.6 ± 0.6 kcal/mol	ΔH = 15.5 ± 1.0 kcal/mol	N/A	D <sub>h</sub> (A3) monomer = 3 nm D <sub>h</sub> (A3-Rep2) = 3.8 nm	N/A	N/A	N/A	N/A

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954 In the last column in grey, previous SPR data obtained by P. Minard's team.\*Rep2

955

956 Table S2. Summary of the results ~~obtained for the interaction Ku-DNA interaction~~ using the  
 957 different techniques.

	AUC-FDS	ITC		SEC-MALS	BLI		MST	switch SENSE		SPR	Diffusion + sizingMDS
DNA(bp)	18*	18	42	42	42**		18*	48	80	200**	18*
Ratio Ku vs DNA	1:1	1:1	2:1	2:1, 1:1	N/A		N/A	1:1		20:1	1:1
K <sub>D</sub> (nM)	12.9 ± 3.2	3.5 ± 0.8	3.76 ± 0.74	N/A	0.4 ± 0.1	5.2 ± 0.2	<del>2.9</del> 11.9	1.0 ± 0.2	3.1 ± 0.1	7.3 ± 3.0	2.5 ± 0.8
k <sub>ON</sub> (M <sup>-1</sup> s <sup>-1</sup> ) k <sub>OFF</sub> (s <sup>-1</sup> )	N/A	N/A	N/A	N/A	1.9 ± 0.1 10 <sup>-6</sup> 7.2 ± 0.3 10 <sup>-4</sup>	N/A	N/A	2.6 ± 0.2 10 <sup>-6</sup> 2.3 ± 0.3 10 <sup>-3</sup>	92.3 ± 0.13 10 <sup>-4</sup> 2.8 ± 0.1 10 <sup>-4</sup>	7.8 ± 4.0 10 <sup>-6</sup> 1.5 ± 0.8 10 <sup>-4</sup>	N/A
Other information	S (DNA) = 2.2 ± 0.1 S (1:1) = 7.3 ± 0.2	ΔH = 10.95 ± 0.1 kcal/mol n = 0.95	ΔH = 243.49 ± 24.79 kcal/mol n = 0.34 ± 0.02	Mw (DNA) = 26.1 kDa Mw (Ku) = 144.4 kDa Mw (1:1) = 171.0 kDa Mw (2:1) = 306.2 kDa	N/A	N/A	N/A	N/A D <sub>h</sub> = 8.9 nm	D <sub>h</sub> = 8.9 nm	N/A	Rh = 7.1 ± 0.2 nm

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958 The last two columns came from additional data: Preliminary SPR obtained by Charbonnier's team, and MDF in  
 959 collaboration with Fluidic Analytics. DNA modified \*FAM or \*\*Biotin.

# 1 **Macromolecular interactions *in vitro*, comparing classical and novel approaches**

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3 Velazquez-Campoy<sup>5,6,7,8,9</sup>, David Stroebel<sup>10</sup>, Guillaume Bec<sup>11</sup>, Pierre Soule<sup>12</sup>, Christophe  
4 Quétard<sup>13</sup>, Christine Ebel<sup>14</sup>, Alain Roussel<sup>15</sup>, Jean-Baptiste Charbonnier<sup>1</sup>, Paloma Fernández  
5 Varela<sup>1#</sup>

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## 25 **Abstract**

26 Biophysical quantification of protein interactions is central to unveil molecular mechanisms of  
27 cellular processes. Researchers can choose from a wide panel of biophysical methods,  
28 including classical and more novel ones. A real-life proof-of-concept was carried out during an  
29 ARBRE-MOBIEU training school held in June 2019 in Gif-sur-Yvette, France  
30 (<https://mosbio.sciencesconf.org/>). Twenty European students benefited from a one-week  
31 training with lessons and practical sessions on six complementary approaches: (i) Analytical  
32 UltraCentrifugation with or without a Fluorescence Detector System (AUC-FDS), (ii) Isothermal  
33 Titration Calorimetry (ITC), (iii) Size Exclusion Chromatography coupled to Multi-Angle Light  
34 Scattering (SEC-MALS), (iv) Bio-Layer Interferometry (BLI), (v) MicroScale Thermophoresis  
35 (MST) and, (vi) switchSENSE. They implemented all these methods on two examples of  
36 macromolecular interactions: firstly, a protein-protein interaction between an artificial  
37 alphaRep binder, and its target protein, also an alphaRep; secondly, a protein-DNA interaction

38 between a DNA repair complex, Ku70/Ku80 (hereafter called Ku), and its cognate DNA ligand.  
39 The students acknowledged that the workshop provided them with a clearer understanding  
40 of the advantages and limitations of the different techniques and will help them in the future  
41 to choose the approaches that are most relevant or informative **for** their projects.

## 42 **Keywords**

43 Molecular scale biophysics, macromolecular interactions, artificial binders, double-stranded  
44 DNA breaks repair factors

## 45 **Introduction**

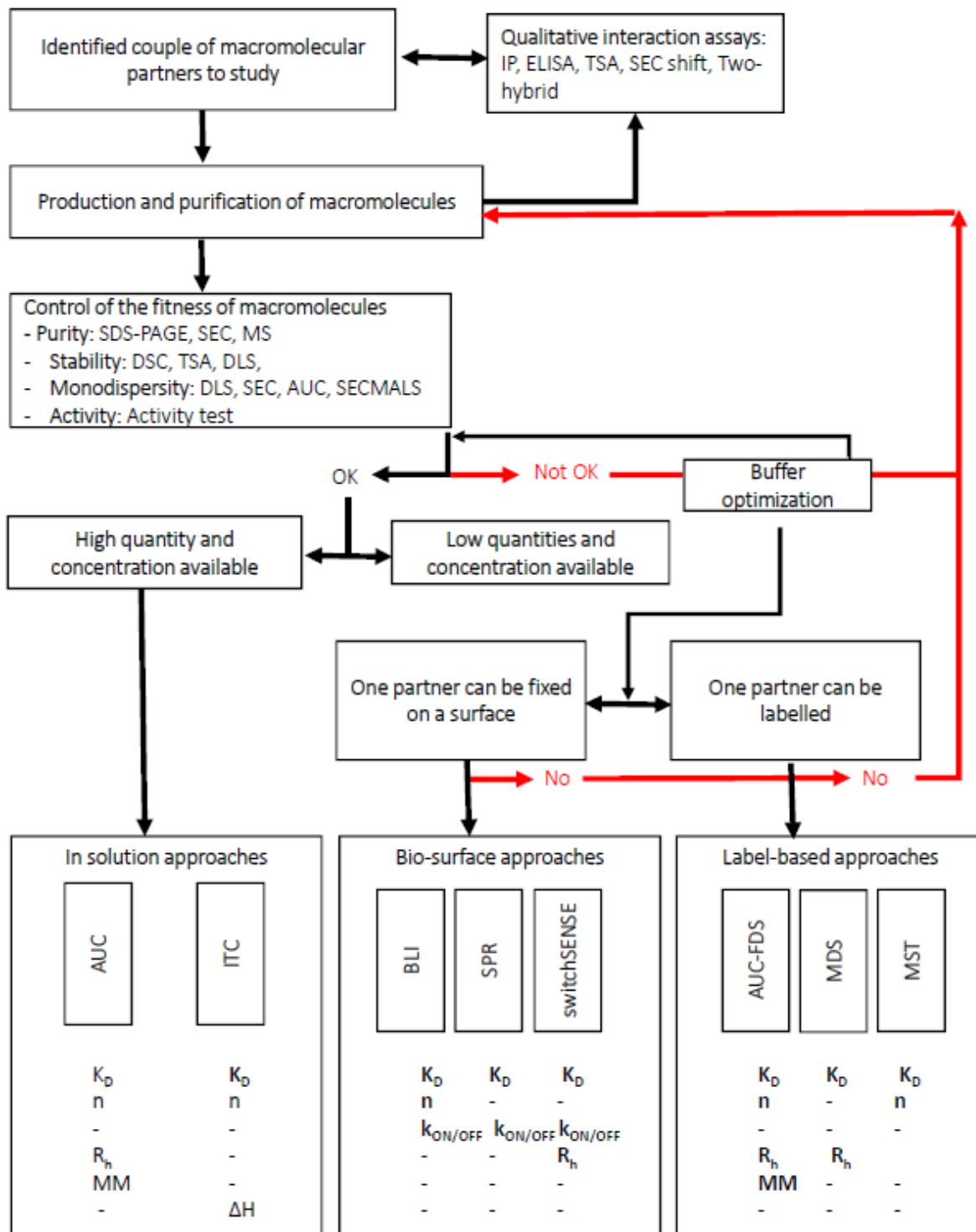
46 Macromolecular interactions play a central role in the activation/inactivation of most cellular  
47 mechanisms. These interactions can be measured *in cellulo*, or *in vitro*, and predicted *in silico*.  
48 The classical *in cellulo* methods (such as tap-tag or two-hybrid) allow large-scale studies, **but**  
49 **the characterization of a direct interaction between two macromolecules relies on the**  
50 **quantitative *in vitro* measurements.** These measurements allow to characterize interactions  
51 not only in terms of affinity, but also to determine additional kinetic and thermodynamic  
52 parameters, as well as to define the hydrophobic or hydrophilic nature of the interface. They  
53 **also** give access to the stoichiometry of the assembly and allow to map the regions involved  
54 by using different constructs or mutants. *In vitro* measurements can also be useful to evaluate  
55 **the drugability of the interaction site or to understand the role of** endogenous regulatory  
56 events on the formation of complexes (**post-translational modifications**). **These**  
57 **measurements are essential to set-up predictive *in silico* docking tools. Conversely, by *in silico***  
58 **analysis,** the structure of macromolecular complexes **as well as** the impact of functional  
59 substitutions **can be predicted,** helping to optimize experimental design (Andreani and  
60 Guerois, 2014).

61 The original idea of the project presented here originated during the organization of a  
62 European Training School in Molecular Scale Biophysics (<https://mosbio.sciencesconf.org/>)  
63 within the MOBIEU COST Action. We proposed to the participants to compare different  
64 techniques allowing to measure macromolecular interactions *in vitro*. Nowadays, there is a  
65 large panel of possibilities, and it becomes difficult to choose which technology will be the  
66 best adapted when embarking into a new project. Each approach presents advantages and



67 drawbacks, and it is therefore difficult for the user to choose from the beginning which one  
68 will be most adapted to the properties of the interaction partners. In the workshop, we  
69 focused on six approaches (Fig. 1). Choosing between the different techniques can be  
70 considered in a progressive manner. If none of the interaction partners can be easily labeled  
71 or immobilized on a surface, approaches in which **both partners** are in solution (AUC, ITC, and  
72 SEC-MALS) should be favored (Fig. 1, top). However, several of them require large quantities  
73 of biological material. When a partner can be immobilized easily on a bio-surface, without  
74 affecting its function, approaches like BLI, switchSENSE and Surface Plasmon Resonance (SPR)  
75 will be often tested, since they offer the possibility to use small amounts of the immobilized  
76 partner (called ligand) (Fig. 1, middle). Finally, when the partners can be labeled, again without  
77 affecting their function, fluorescent probes can be grafted allowing the use of reduced  
78 amounts of material and facilitated signal analysis (AUC FDS, MST, or Microfluidic diffusional  
79 sizing (MDS)) (Fig. 1, bottom). The choice of the optimal approach may further rely on  
80 additional criteria including the solubility of the partners, the instrument environment or non-  
81 specific interactions with instrument surfaces. Finally, it should be stressed that it is preferable  
82 to perform measurements using several orthogonal techniques to fully validate and  
83 characterize a biological interaction, and specify its features, such as stoichiometry, kinetics  
84 or thermodynamics.

85 **Figure 1. Decisional tree to help choosing the biophysical approach **best suited for** the study**  
86 **of a specific molecular interaction.**



87

88 To determine whether two macromolecular partners potentially interact, first indication of a binding event could  
 89 be obtained with qualitative methods (top right): Immunoprecipitation (IP) from cell extracts; Thermal Shift Assay  
 90 (TSA); Size Exclusion Chromatography (SEC) shift, measuring the elution volume change after complex formation;  
 91 Two-hybrid assay, *in cellulo*. The production and purification of the macromolecular partners should aim at  
 92 reaching high purity, time-stability and monodispersity of the preparation for the interaction measurements to  
 93 be reliable. Mass-Spectrometry (MS); Differential Scanning Calorimetry (DSC); TSA; Dynamic Light Scattering  
 94 (DLS); Activity test (for instance an enzymatic assay); Analytical UltraCentrifugation (AUC); Size Exclusion  
 95 Chromatography coupled with Multi-Angle Light Scattering (SEC-MALS). Macromolecular interaction methods  
 96 can be classified in three main groups: measurements in solution and label free (AUC; Isothermal Titration

97 Calorimetry (ITC) (bottom, left)), on biosensor with a partner graft on a surface (Bio-Layer Interferometry (BLI);  
98 Surface Plasmon Resonance (SPR); switchSENSE technology (bottom, middle)), and methods with a partner  
99 labeled with a fluorescent probe (AUC-Fluorescence Detector System (AUC-FDS); Microfluidic Diffusional Sizing  
100 (MDS); MicroScale Thermophoresis (MST) (bottom, right)). These approaches give access to different parameters  
101 of the interaction and present some specific limitations. If a high quality sample (pure, stable, monodisperse) is  
102 available in large quantity (up to mg amount) one may start with label-free and in solution approaches.  
103 Otherwise, if material is limited for one partner, surface and labeling approaches are a good alternative and  
104 highly complementary to cross-validate interaction measurements. Surface approaches are best candidates to  
105 get access to the kinetic parameters of interaction. Six of these approaches were used in the training school.  
106 Dissociation constant ( $K_D$ ); stoichiometry ( $n$ ); association and dissociation rates ( $k_{ON}$ ,  $k_{OFF}$ ); hydrodynamic radius  
107 ( $R_h$ ); Molecular mass (MM).

108 In this training school, we used as examples two different macromolecular interactions  
109 systems, that have been well characterized in our laboratories using several of the approaches  
110 discussed here. One is an interaction between two proteins, and the other a protein-DNA  
111 interaction, both with an affinity in the nanomolar range. Reference data were initially  
112 produced in our laboratories. A group of 20 participants reproduced our measurements during  
113 the five-day MoSBio Training School.

114 The first project comes from P. Minard's team, who uses an original family of artificial  
115 helicoidal repeat proteins, called alphaRep (Guellouz et al., 2013). AlphaRep libraries allow to  
116 select tight binders against a variety of targets by phage display. The alphaRep's are highly  
117 soluble proteins, easily expressed in *E. coli*, which display a very high thermal stability. These  
118 proteins are cysteine-free and, thus do not contain disulfide bonds. They are composed by  
119 repeated motifs made with two antiparallel alpha helices. Clusters of variable side chains,  
120 mainly in the second helix, are positioned on the same face of the motifs. The ensemble of all  
121 these variable motifs forms a library of surfaces from which tight binders can be extracted  
122 against a given target. The alphaRep's have been used for several applications, such as  
123 chaperones for crystallization and structural studies of difficult targets (Valerio-Lepiniec et al.,  
124 2015; Di Meo et al., 2017; Chevrel et al., 2018; Campanacci et al., 2019), as well as in  
125 biophysical and live cell studies (Léger et al., 2019; Prasad et al., 2019; Fernandez et al., 2020;  
126 Léger et al., 2020). New applications of these artificial binders are currently being explored in  
127 relation with their ability to be expressed in eukaryotic cells. Here we analyzed the interactions  
128 between two alphaRep's (Rep2 and Rep17) selected against a protein target, (A3), which is  
129 itself an alphaRep. The interaction between the alphaReps has been extensively characterized  
130 before in the laboratory by ITC, SEC-MALS and AUC, but also by Circular Dichroism (CD), SPR  
131 and Fluorescence Resonance energy Transfer (FRET) (not used in this training school)  
132 (Guellouz et al.,2013; Di Meo et al., 2017; Léger et al., 2019). Both A3 / Rep2 and A3 / Rep17  
133 interactions were tested previously to determine which one was the most appropriate for the  
134 training. Because the former is weaker than the latter, we focused only on the only A3 / Rep17  
135 interaction during the training.

136 The second project comes from J.B. Charbonnier's team. It concerns proteins involved in DNA  
137 repair, and more precisely, in the classical Non-Homologous End Joining (c-NHEJ), the main  
138 Double-Strand Break (DSB) repair pathway in human. The Ku70/Ku80 (Ku) heterodimer plays

139 a central role in the recognition of DSB ends, as it is the first repair factor that interacts with  
140 them. Ku binds tightly to DNA ends in a sequence independent manner thanks to its ring-  
141 shaped structure (Walker et al., 2001). Ku then iteratively recruits different NHEJ partners  
142 (ligase 4, nucleases and polymerases) (Chang et al., 2017; Frit et al., 2019). Ku also contributes  
143 to the tethering (synapse) between the two DSB ends to avoid misrepair with other DSB ends.  
144 J.B. Charbonnier's team (Tadi et al., 2016; Nemoz et al., 2018) has recently described the  
145 recruitment mechanism of some NHEJ factors by Ku at the molecular level. DSBs, despite being  
146 deleterious DNA lesions, are generated on purpose during radiotherapy or in genome editing  
147 by CRISPR-Cas9. Understanding the molecular basis of the c-NHEJ is thus central to improve  
148 these major biotechnological applications. Here we analyzed the interaction between Ku and  
149 DNA substrates of different lengths, to determine the most appropriate for the training and  
150 to study the threading of Ku on DNA. The characterization of the interaction between Ku and  
151 DNA **has been** extensively studied in the laboratory by ITC, MST (Tadi et al., 2016; Nemoz et  
152 al., 2018; Gontier **et al., 2021**), switchSENSE, AUC, BLI (data not published) and, more recently  
153 by other techniques not used during the training school (SPR, MDS or FRET). One Ku occupies  
154 about 18 bp and several Ku molecules can thread on DNA when **its** size is longer than 18bp,  
155 **with** one Ku molecule every 18bp.

156 We present in this article the results obtained by 20 students during a European Training  
157 School in Molecular Scale Biophysics that took place from June 3<sup>rd</sup> to 7<sup>th</sup>, 2019 at I2BC at Gif-  
158 sur-Yvette, France. We report the protocols used to analyze the two systems under study  
159 (protein/protein and protein/DNA interactions) using the six biophysical methods mentioned  
160 above. The results obtained by the students during **the one-week course** are shown and  
161 compared to our previous published data when available. Finally, we compare the advantages  
162 and drawbacks of the different approaches used during this training **school** and present some  
163 **feedback** from the students, allowing to have a global overview of the pros and cons of these  
164 six complementary biophysical approaches.

165 **Materials and Methods**

166 **Biophysical approaches in solution that do not require labeling of an interaction partner**

167 **Size Exclusion Chromatography with Multi-Angle Laser Light Scattering detection (SEC-**  
168 **MALS):** SEC-MALS allows to determine the absolute molar mass of the components **present in**  
169 **a macromolecular** sample. It indicates if elution peaks are homogenous or if they are  
170 composed of mixtures, either of different oligomers or of different conformers. SEC-MALS  
171 allows size determination if an online Dynamic Light Scattering (DLS) detector is included in  
172 the setup (Folta-Stogniew, 2006). **In order to determine stoichiometry in a macromolecular**  
173 **hetero-complex, differences in extinction coefficients are required. An experimental**  
174 **determination of extinction coefficient by analyzing each partner separately, will be more**  
175 **precise than *in-silico* prediction. For a more precise estimation of molar mass of complex**  
176 **components, you need to know the refractive index increment ( $dn/dc$  value) of each complex**  
177 **partner at the wavelength of the MALS system laser.** We used an HPLC system from *Shimadzu*  
178 coupled to a MALS detector (miniDAWN TREOS) plus a DLS detector (QELS), and a  
179 refractometer (optilab T-rEX) from *Wyatt technologies*. To run an experiment, one needs 1 L  
180 of running buffer to equilibrate the column and detectors, **from 2 mg/mL** of protein (for MW  
181 of 20 kDa), down to 0.5 mg/mL (if the MW is higher than 150 kDa). Depending **on** the column  
182 used, the volume of the sample (30  $\mu$ L for Bio-SEC-3 *Agilent*, 50  $\mu$ L for KW-803/804 *Shodex*,  
183 and 100  $\mu$ L for Superose 6, Superdex 75 or Superdex 200 increase 10/300 GL *Cytiva*) and the  
184 time of elution can differ. A run (including an equilibration step and a control BSA sample)  
185 takes typically a few hours and consumes 40-200  $\mu$ g of sample.

186 **Isothermal Titration Calorimetry (ITC):** ITC allows the direct, and thorough thermodynamic  
187 characterization of interactions between molecules in solution with no limitation of partner  
188 size **and without labeling** (Holdgate, 2001; Krell, 2008; Velazquez-Campoy and Freire, 2006).  
189 ITC **allows** to quantify dissociation constants ( $K_D$ ), but also other interaction parameters  
190 (enthalpy, entropy, stoichiometry, and heat capacity). ITC is not affected by the optical  
191 properties of the samples, but may be very sensitive to the composition of the buffer (e.g **in**  
192 **particular to** mismatches between **partner sample** solutions). **In a microcalorimeter the two**  
193 **cells (reference and sample) needs to be kept at exactly the same temperature. A heat sensing**  
194 **device detects temperature difference between the cells when binding occurs, upon serial**  
195 **injection of a ligand, and give feedback to the heaters, which compensate for this difference**

196 and return the cells to equal temperature. Each injection gives then rise to a peak of emitted  
197 or absorbed heat whose surface is proportional to the amount of binding. For the ITC  
198 experiments, we used three instrument models: VP-ITC, ITC200 and PEAQ-ITC all from  
199 *Malvern Panalytical*. One of the interacting partners is placed in a cell (1.4 mL for the VP-ITC,  
200 200  $\mu$ L for the ITC200 and PEAQ-ITC) and the other in a syringe (300  $\mu$ L for the VP-ITC, 40  $\mu$ L  
201 for the ITC200 and PEAQ-ITC). Sequential injections are made from the syringe (5-10  $\mu$ L for  
202 the VP-ITC, 1-2  $\mu$ L for the ITC200 and PEAQ-ITC) into the cell. To produce the data reported  
203 here, we used 80-990  $\mu$ g of the molecule in the cell, and 130-320  $\mu$ g of the molecule in the  
204 syringe. The transient heat effect due to complex formation (and other potential unspecific  
205 phenomena) upon partner injection is measured as the titration progresses, from which the  
206 binding isotherm is constructed.

## 207 **Biophysical approaches with a partner immobilized on a surface**

208 **Bio-Layer Interferometry (BLI):** BLI is a fast, high throughput and label-free technology for  
209 measuring biomolecular interactions by analyzing the interference pattern of white light  
210 reflected from a layer of immobilized macromolecules on a biosensor tip versus an internal  
211 reference (Abdiche et al., 2018). It enables real-time determination of affinity, kinetic  
212 parameters and concentration, with one of the binding partners immobilized onto the  
213 biosensor surface (ligand) and the other in solution (analyte). This microfluidic-free technology  
214 is particularly adapted for performing binding assays in crude lysates or cell culture media. We  
215 used an Octet RED96e during the training, and an RED384 during the preparation phase, both  
216 from *FortéBio*. In all cases, one of the interaction partners will be non-covalently captured on  
217 a glass sensor probe. The sensor will then be incubated in microplate wells containing the  
218 second interaction partner in solution. A light beam is sent through the sensor, and the  
219 reflected beam is analyzed using an interferometric detector, that measures the spectral shift  
220 due to the variations of density (optical thickness) that the bilayer undergoes upon  
221 association and dissociation of the complex. The real-time monitoring of these variations  
222 allows to record a « sensorgram », from which the kinetic rates ( $k_{ON}$  and  $k_{OFF}$ ), as well as the  
223 equilibrium constant  $K_D$  can be determined through mathematical curve fitting. We used 1-5  
224  $\mu$ g/mL of ligand to load NTA sensors for 20-120 s, for low density, or 120-600 s, for high  
225 density, and a range of at least 7 2-fold dilutions (starting at 100-200 nM) of the analyte with  
226 an association time of 600-900 s. We consumed about 8  $\mu$ g of protein A3, and 400 ng of 42 bp

227 biotinylated DNA **per run** for the immobilization, and about 2-14  $\mu\text{g}$  of **the** respective analytes.  
228 The concentration of ligand could be reduced (at least by 2) in favor of a longer incubation.  
229 The consumption of the analyte, depends on the affinity of the interaction, as the  
230 concentrations used should range from  $K_D/10$  to  $10 \times K_D$ .

231 **switchSENSE**: switchSENSE technology is based on short DNA nanolevers (48 bp in our case),  
232 which are immobilized on gold electrodes in a microfluidic channel. The intrinsically negatively  
233 charged DNA nanolevers can be electrically actuated (“switched”) on the gold surface to  
234 oscillate at high frequencies (Knezevic et al., 2012). A switchSENSE microfluidic biochip  
235 contains four flow channels, each containing six gold electrodes. Switching of the DNA is  
236 mediated by alternating the voltage across the gold surface. **The motion of the levers is**  
237 **tracked in real time ( $\mu\text{s}$  scale) via electrically triggered time correlated single photon counting**  
238 **(E-TCSPC) detecting a fluorescent probe (dye) present on top of the immobilized DNA strands.**  
239 **The recorded fluorescence intensity correlates to the orientation of the DNA nanolever**  
240 **relative to the surface as the fluorescence is gradually quenched upon approaching the gold**  
241 **electrode due to energy transfer.** The complementary DNA strands can be cross-linked to a  
242 ligand via amine or thiol coupling or click-chemistry. By hybridization of this conjugated  
243 complementary strand to the surface-tethered DNA nanolever, the surface is functionalized  
244 with the ligand of interest. Upon binding of an analyte, the hydrodynamic friction of the levers  
245 is **increased** and subsequently the movement of the levers is slowed down. This change in  
246 switching speed is used by the system to determine the size ( $D_h$ ) or conformational changes  
247 of ligands and complexes. The kinetics of molecular interactions ( $k_{ON}$ ,  $k_{OFF}$ ,  $K_D$ ) can be  
248 **monitored** using two measurement modes: dynamic or static. In the first case, analyte binding  
249 is measured through the change of the oscillation rate of the electrically actuated DNA  
250 nanolevers (changes in dynamic response). In the second case, the DNA nanolevers are kept  
251 at an upright position, in a constant electric field, and analyte in close proximity to a dye can  
252 alter **its** local chemical environment resulting in a fluorescence change (also called  
253 Fluorescence Proximity Sensing). Binding is then measured thanks to the fluorescence  
254 intensity variation of the functionalized nanolever. For the switchSENSE experiments, we used  
255 a DRX<sup>2</sup> device from *Dynamic Biosensors* with two LED light sources (for the excitation of red  
256 and green fluorophores). In all cases, a hundred  $\mu\text{g}$  of protein were enough to generate cross-  
257 linked complementary strands (cNL-DNA) for several rounds of experiments, since one



258 measurement needs only 40  $\mu\text{L}$  of 100 nM DNA-protein conjugate. A sizing measurement  
259 classically takes less than an hour. The **quantity** of analyte needed for a kinetic experiment  
260 depends on the overall affinity, **the** flow rates, and **the** association time. **A** too slow flow rate  
261 can **result in** mass transport limitation during **association and** in re-binding effects **during**  
262 **dissociation**. Here we used flow rates of 100-500  $\mu\text{L}/\text{min}$ , association times of 80-300 s, and  
263 1500 s of dissociation time. We used 150 ng of the cross-linked ligands, and 2-20  $\mu\text{g}$  of its  
264 partner for a series of 3 concentrations.

## 265 **Biophysical approaches with a labeled partner**

266 **Analytical UltraCentrifugation with Fluorescence Detection (AUC-FDS):** AUC is a powerful  
267 technique for the characterization of macromolecules and macromolecular self- and hetero-  
268 association processes in solution. It was used here with labeled protein or DNA, but it can be  
269 used with non-labeled material like SEC-MALS and ITC. An analytical ultracentrifuge is a high-  
270 speed centrifuge equipped with one or more detectors (absorbance/interference,  
271 fluorescence) allowing to monitor sedimentation in real time. Two types of complementary  
272 experiments can be performed, sedimentation velocity and sedimentation equilibrium (Zhao  
273 et al., 2013). Sedimentation velocity experiments allow to determine the size distribution of  
274 species, their aggregation and oligomerization, sedimentation coefficients, hydrodynamic  
275 radius, shape and molar masses, their stoichiometry and  $K_D$  (by isotherm fitting **of**  
276 sedimentation coefficients measured at various concentrations). Sedimentation equilibrium  
277 **experiments are** suited for well-defined samples, and **give** molar mass and  $K_D$  information. The  
278 centrifugation speed can be set between 600 and 260 000 g allowing to study all sizes of  
279 macromolecules. The duration of sedimentation experiments ranges from 2 h to several days.  
280  **$dn/dc$  and UV extinction coefficients values, which are needed to analyse data obtained with**  
281 **absorbance and interference optics, can be determined from the protein sequence using the**  
282 **SEDFIT calculator module (Schuck, 2000).** For the AUC-FDS experiments we used a  
283 ProteomeLab XL-I ultracentrifuge from *Beckman Coulter* equipped with a Fluorescence  
284 Detection System (FDS) from *AVIV Instruments*. A sedimentation velocity experiment requires  
285 100-450  $\mu\text{L}$  of sample at 0.3 to 1.5 OD, for absorbance/interference detection, and 5-60 nM  
286 concentration, for fluorescence detection, whereas sedimentation equilibrium requires 130  
287  $\mu\text{L}$  at 0.2 to 0.5 OD. In interference, the limit of detection is 0.1 mg/mL, but in absorbance it  
288 depends on **the** sample extinction **coefficient. To characterize an interaction using the AUC**

289 fluorescence detector, a range of 2 nM to 2  $\mu$ M is typically used. When the affinity is very high,  
290 a range between 2 pM and 2 nM is chosen, but a blocking agent - e.g. BSA at 1 mg / mL - is  
291 added in the sample to avoid non-specific adsorption and loss of fluorescent dye. To produce  
292 the data described here, we used about 1-3.5  $\mu$ g of the fluorescent molecule and 8-80  $\mu$ g of  
293 the non-fluorescent molecule.

294 **Microscale Thermophoresis (MST):** MST is a novel technology for the analysis of biomolecules  
295 based on the modification of fluorescence intensity induced by the temperature, due to  
296 Temperature Related Intensity Changes (TRIC) and the directed movement of particles in a  
297 microscopic temperature gradient (thermophoresis) (Asmari et al., 2018; Jerabek-Willemsen  
298 et al., 2011). Thermophoresis is influenced by a combination of changes at the level of the  
299 hydration shell, shape, charge..., of biomolecules (all the parameters that influence the Soret  
300 coefficient), which result in differences of movement along the temperature gradient as well  
301 as of brightness of the fluorescent tag. MST provides information on the binding affinities with  
302 good accuracy and sensitivity in the pM to mM range. This technology allows immobilization-  
303 free measurement of interactions in any buffer and complex biological liquid, but requires one  
304 of the two partners to be labeled with a fluorescent dye to measure protein-protein  
305 interactions (there is also a label free instrument that detects the intrinsic fluorescence of  
306 proteins). Any size of unlabeled molecules can be used (from ions, to large proteins). **MST**  
307 **experiments are performed in capillaries, thus require a low sample consumption. Method**  
308 **development for MST is usually fast and consists of three steps: (i) ensure there is enough**  
309 **fluorescence signal intensity. (ii) Check your sample for sticking to the capillaries. (iii) Establish**  
310 **the noise floor of your experiment by measuring MST of just your target without ligand at the**  
311 **final settings (final concentration, excitation laser and « MST power » = IR laser LED power to**  
312 **be used).** We used a Monolith NT.115 blue/green and a red/blue from *NanoTemper*  
313 *Technologies*, 200  $\mu$ L at 20 nM of the fluorescent molecule and 20  $\mu$ L of the non-fluorescent  
314 molecule at the highest concentration needed (depending on the expected  $K_D$ ). To produce  
315 the data described here, we used about 50-350 ng of the fluorescent molecule and 6-12  $\mu$ g of  
316 the non-fluorescent molecule.

### 317 **Sample preparation**

318 To perform biophysical characterization measurements, samples of high purity, stability and  
319 monodispersity are needed (Fig. S1). We chose well characterized systems from our

320 laboratories, and the concentration ranges employed to accurately determine binding  
321 parameters were already known.

322 AlphaReps are recombinant proteins produced by standard overexpression procedures in  
323 *E.coli* (Guellouz et al., 2013). For our experiments, we used the following alphaRep proteins:  
324 A3, which is the target and Rep17 and Rep2, which are the binders. Because A3 forms  
325 homodimers at high concentration, it was used as ligand for real-time biosensors approaches  
326 (BLI, switchSENSE) and was the labeled partner in AUC-FDS and MST experiments. In the  
327 conditions used, we assumed A3 is a monomer. The simultaneous presence of the monomeric  
328 and dimeric forms would make the analysis difficult. A3 dimers are unable to interact with  
329 Rep17, and it is only when they dissociate into monomers that, the interaction with Rep17  
330 occurs. A dissociation constant of 37 nM for the A3 / A3 dimer was measured by AUC (Léger  
331 et al., 2019). This is why we observe several events in ITC: the dissociation of the A3 dimer and  
332 the interaction with Rep17.

333 Ku is a recombinant protein produced by standard overexpression procedures in insect cells  
334 (Nemoz et al., 2018). For our experiments, we used different lengths of dsDNA depending on  
335 the assay format. Shorter DNA are not adapted for biosensor approaches, since a DNA too  
336 close to the surfaces will hinder its interaction with Ku. DNA is practical to work with, because  
337 it is easy to modify and is commercially available. For immobilization on BLI biosensors and for  
338 detection in AUC-FDS and MST, we ordered biotinylated and 5-Carbofluorescein (5-FAM)-  
339 labeled DNA oligonucleotides, respectively.

340 All proteins were dialyzed to eliminate glycerol from the storage buffer, which can interfere  
341 with the measurements. We chose the pH and ionic strength of the buffer for the best  
342 solubility of the samples. To simplify as much as possible, we used the dialysis buffer (20 mM  
343 Tris-HCl pH 8, 150 mM NaCl, 5 mM  $\beta$ -mercaptoethanol) as running buffer in most experiments.  
344 Due to the particularities of each approach and measurement devices, there are some  
345 limitation in the buffer choice. In some cases, blocking agents were required, such as 1mg/mL  
346 BSA, especially for low protein concentrations (in the nM range), to prevent surface  
347 adsorption. To reduce surface tension in capillaries and microfluidic devices, detergents were  
348 required, such as 0.1 % Tween-20. For AUC-FDS, we had to avoid Tris-HCl or HEPES above 20  
349 mM, which can cause problems at 230 nm wavelength, and be aware of other absorbent

350 molecules (nucleotides, old DTT or  $\beta$ -mercaptoethanol). For MST experiments, we used the  
351 commercial MST buffers (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 10 mM MgCl<sub>2</sub>, 0.05 % Tween-  
352 20) for Ku-DNA and, Roti<sup>®</sup>-Stock 1x PBS (10 mM Na<sub>2</sub>HPO<sub>4</sub>, 2 mM KH<sub>2</sub>PO<sub>4</sub> pH 7.6 137 mM NaCl,  
353 2.7 mM KCl, 0.005% Tween 20) for A3-Rep17. For switchSENSE sizing experiments D<sub>h</sub>  
354 estimation, a low salt buffer is required (10 mM Tris-HCl, 40 mM NaCl, 0.05 % Tween-20, 50  
355  $\mu$ M EGTA, 50  $\mu$ M EDTA).

356 In order for new users to determine the concentration ranges to work with in an unknown  
357 system, preliminary experiments need to be done using a broad range, observe the results  
358 and in consequence refine the initial conditions to a narrow range that could better suit the  
359 system in question. If an interaction partner is difficult to produce or not stable at high  
360 concentration, in solution approaches will be the one to keep in constant concentration during  
361 titration of its partner. In the other approaches must be the one to try first to immobilize or  
362 label. Reverse configuration can be always tested to improve or confirm the results.

## 363 Results

### 364 Biophysical measurements of alphaRep interactions

365 We characterized the interaction of A3 with its binder Rep17 (Rep2 was also used during the  
366 preparation of the training) using the following techniques presented in the Fig. 1: in solution  
367 approaches, which do not require labeling (SEC-MALS and ITC), in solution approaches that  
368 require labeled protein or DNA (MST and AUC-FDS), and surface approaches (BLI and  
369 switchSENSE). The students performed the measurements presented here during the training  
370 (Fig. 2, Fig. S2 and Table S1). When available, we mention the values that have been reported  
371 for some approaches in previous studies.

### 372 AlphaRep interactions measured by label free in solution approaches

373 Due to time limitations, the SEC-MALS experiments were performed prior to the training. We  
374 used a Superdex 200 increase 10/300 GL column (*Cytiva*), and compared three different runs  
375 with A3 alone, Rep2 alone and A3-Rep2 complex at 2 mg/mL each. A3 eluted as a dimer with  
376 a molar mass of  $44.8 \pm 0.4$  kDa and, a hydrodynamic radius of  $3.0 \pm 0.2$  nm. Rep2 eluted as a  
377 monomer with a molar mass of  $11.6 \pm 0.9$  kDa. The Rep2 concentration was insufficient, in  
378 view of the small molar mass, to make accurate sizing measurement by DLS. Finally, the A3-  
379 Rep2 complex eluted before the free proteins, with a molar mass of  $65.5 \pm 0.4$  kDa and  
380 hydrodynamic radius of  $4.0 \pm 0.2$  nm (Fig. 2b). The stoichiometry was determined by using the  
381 protein conjugate method (Loiseau et al., 2017). Accurate mass can be calculated from MALS  
382 data with this method, which uses two concentration detectors simultaneously (a  
383 refractometer and UV spectrophotometer), and information about refractive index increment  
384 ( $dn/dc$ ) and UV extinction coefficient of each component. In our case, the complex  
385 corresponds to one dimer of A3 and two Rep2 molecules.

386 Preparatory ITC experiments were performed on a PEAQ-ITC instrument with A3 in the cell at  
387  $18 \mu\text{M}$  and Rep17 in the syringe at  $187 \mu\text{M}$  (Fig. S2a). Three different groups of students did a  
388 triplicate measurement during the training using the same PEAQ-ITC instrument. We observed  
389 bi-phasic thermograms, and isotherms were fitted using a model of two identical binding sites  
390 with cooperativity. This cooperativity could stem from the propensity of A3 to form  
391 homodimers at  $\mu\text{M}$  concentration as shown by SEC-MALS (Freire et al., 2009; Vega et al.,  
392 2015). We could estimate two  $K_D$  values, the first  $K_D$  corresponds to an interaction with a tight

393 affinity ( $K_{D1}$  from 0.48 to 0.66 nM) and the second one to a weaker affinity ( $K_{D2}$  from 16 to 32  
394 nM) (Fig. 2c). With the obtained parameters for each site, described in the table at the bottom  
395 of thermograms, an average global affinity (Wyman and Gill, 1990) could be calculated  
396 (geometric mean of both dissociation constants:  $K_{D,av} = \sqrt{K_{D1} \times K_{D2}}$ ), which for each assay  
397 was 3.9 nM, 3.1 nM, and 3.2 nM, close to that obtained with other techniques. Data were  
398 analyzed during the training using the Origin software and the new PEAQ ITC software for  
399 comparison.

#### 400 **AlphaRep interactions measured by biophysical approaches with a partner immobilized on** 401 **a surface**

402 Preliminary analysis using SPR, were carried out a ProteON XPR36 instrument from *Biorad*  
403 (data not published, Fig. S2b).

404 **Before the training** preliminary BLI experiments were performed **on an Octet RED384**  
405 instrument where His-tagged A3 protein was captured on Ni-NTA sensors at 5  $\mu\text{g}/\text{mL}$  for 20 s  
406 and Rep17 concentration **ranged** from 200 nM to 1.56 nM. In these conditions, in which A3 is  
407 most probably in a monomeric form, the calculated kinetic rates were  $k_{ON}$  of  $1.3 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$ ,  
408  $k_{OFF}$  of  $5.9 \times 10^{-3} \text{ s}^{-1}$  and  $K_D$  of 22.4 nM (data not shown). We observed that the fit was not correct  
409 with a single site model and that residuals showed systematic errors. During the training, a  
410 duplicate experiment was performed on an Octet RED96e in a buffer containing 1 mg/mL BSA  
411 and 0.1 % Tween-20 to limit the non-specific binding. In this case, the fitting was improved  
412 (Fig. 2d). We obtained the following preliminary values:  $k_{ON}$  of  $2.6 \pm 0.7 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$ ,  $k_{OFF}$  of  $7.6$   
413  $\pm 0.7 \times 10^{-4} \text{ s}^{-1}$ ,  $K_D$  of  $3.2 \pm 1.1 \text{ nM}$ . The  $K_D$  measured from the plateau of the curve (steady state)  
414 was of  $3.3 \pm 0.8 \text{ nM}$ , close to the one obtained from kinetics (Fig. S2c).

415 For the switchSENSE experiments, we first coupled A3 with the DNA strand (cNL-B48), which  
416 was complementary to the surface-tethered DNA strand on the chip. In this case, the surface-  
417 tethered DNA strand was labeled with a red fluorescent probe (NLB48-red dye). The DNA-  
418 protein conjugate was purified using an anion-exchange-chromatography (Fig. S2d, top left).  
419 We then hybridized the A3-cNLB48 with the NLB48 on the sensor surface at a concentration  
420 of 100 nM. The hybridization step could be monitored in real-time by measuring the  
421 fluorescence increase (Fig. S2d, top right). Preliminary kinetic experiments were done using  
422 the dynamic mode (switching of the nanolevers). Rep2 alphaRep was used as analyte at three

423 different concentrations ranging from 100 nM to 11.1 nM. Preliminary kinetic rates were  
424 calculated from a global fit for the three concentrations:  $k_{ON}$  of  $2.40 \pm 0.05 \cdot 10^6 \text{ M}^{-1}\text{s}^{-1}$ ,  $k_{OFF}$  of  
425  $6.86 \pm 0.23 \cdot 10^{-4} \text{ s}^{-1}$  and  $K_D$  of  $0.29 \pm 0.01 \text{ nM}$  (Fig. S2d, bottom, left). For each analyte  
426 concentration, it was possible to perform a sizing experiment to measure the hydrodynamic  
427 diameter of the ligand A3, before or after association with Rep2. The  $D_h$  of the dimeric form  
428 of A3 calculated from the crystal structure (PDB 6FT) is found to be equal to 4.8 nm. Due to  
429 protein dilution, we expect A3 to be present as a monomer on the DNA conjugated strand.  
430 Indeed, the  $D_h$  of  $3.0 \pm 0.1 \text{ nm}$  measured in our switchSENSE experiments is compatible with  
431 a monomeric form of A3 (Fig. S2d, bottom, right). The  $D_h = 3.8 \pm 0.1 \text{ nm}$  measured for the  
432 complex A3-Rep2 corresponds to an increase of 0.8 nm when compared to the hydrodynamic  
433 diameter of the monomeric form of A3 (Fig. S2d, bottom, left). During the training, kinetic  
434 experiments in static mode (nanolevers in up position) were performed, **as before for Rep2,**  
435 **with Rep17 concentration ranging from 100 nM to 11.1 nM.** The calculated kinetic values  
436 measured in this case were in the same range than the ones obtained previously **with Rep2:**  
437  $k_{ON}$  of  $2.35 \pm 0.17 \cdot 10^6 \text{ M}^{-1}\text{s}^{-1}$ ,  $k_{OFF}$  of  $2.30 \pm 0.34 \cdot 10^{-3} \text{ s}^{-1}$  and  $K_D$  of  $0.98 \pm 0.16 \text{ nM}$  (Fig. 2e).  
438 During the training, students did not have time to run a sizing experiment.

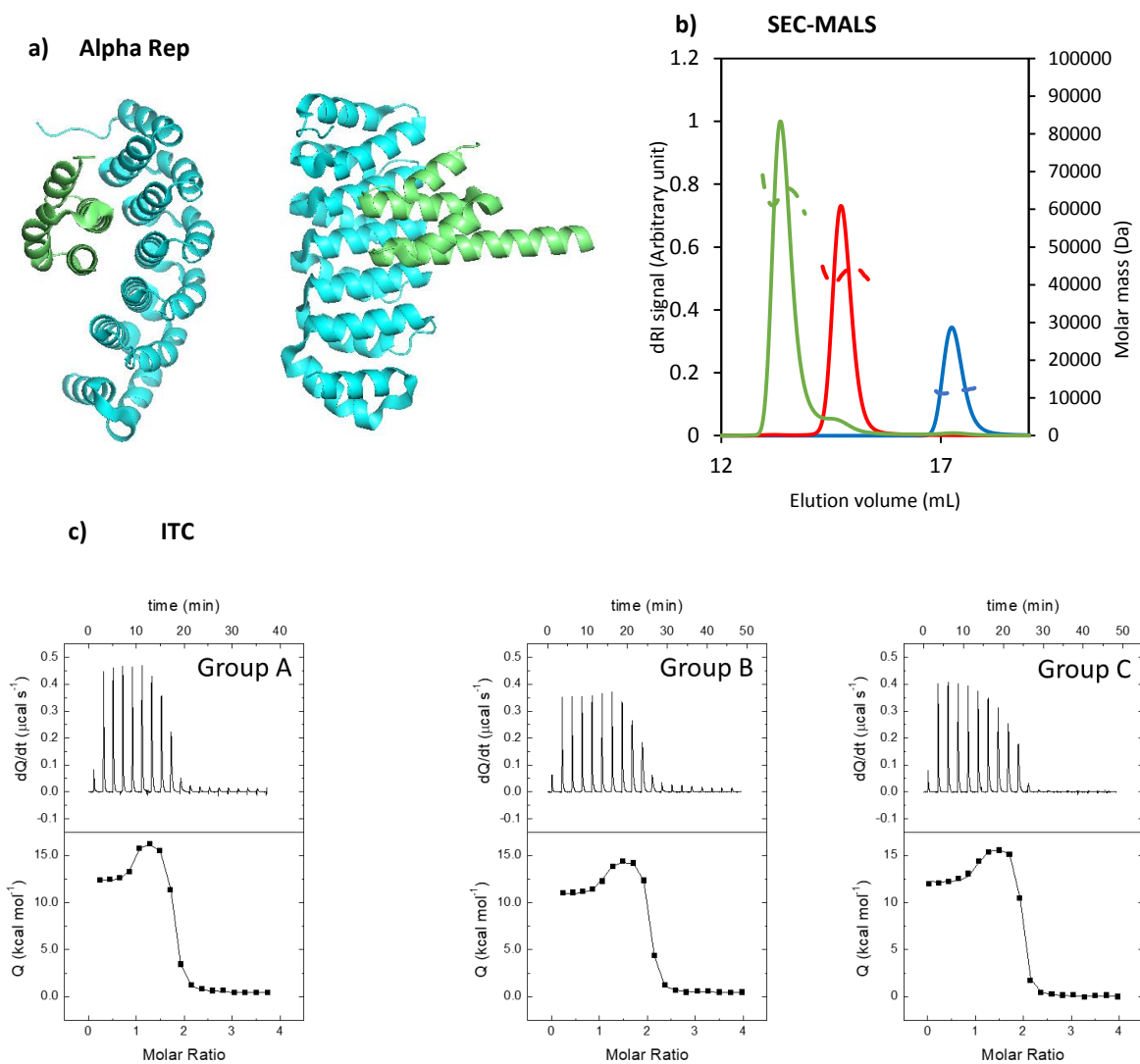
#### 439 **AlphaRep interactions measured by biophysical approaches with a labeled partner**

440 Because AUC-FDS experiment **was** time consuming, data were collected before the training.  
441 We first labeled A3 with the dye NT495 using the commercial *NanoTemper* Monolith Protein  
442 Labeling Kit BLUE-NHS (Amine Reactive). In these conditions, we were able to use a low  
443 concentration of A3 (5 nM). At this nanomolar concentration, A3 is a monomer as observed  
444 before by switchSENSE. We used variable concentrations of Rep17 from 0.5 nM to 0.5  $\mu\text{M}$ ,  
445 and an An-50 Ti rotor at 42 000 rpm (130 000 g) speed. The sedimentation coefficients for A3  
446 and A3-Rep17 complex were  $2.16 \pm 0.08 \text{ S}$  and  $2.96 \pm 0.05 \text{ S}$ , respectively and the calculated  
447  $K_D$  at equilibrium was  $13.4 \pm 1.8 \text{ nM}$  (Fig. 2f).

448 Finally, for the MST experiment, we decided to use **the Red-tris-NTA 2<sup>nd</sup> Generation dye from**  
449 **the *NanoTemper* Monolith His-Tag Labeling Kit**, this site-specific non covalent labeling  
450 substantially improved the signal **respect to the 1<sup>st</sup> Generation dyes**, and respected better the  
451 integrity of protein. **Red-tris-NTA labeled A3 could not be used in AUC-FDS experiment,**  
452 **because its excitation and emission maxima are out of the fluorescence detector range**

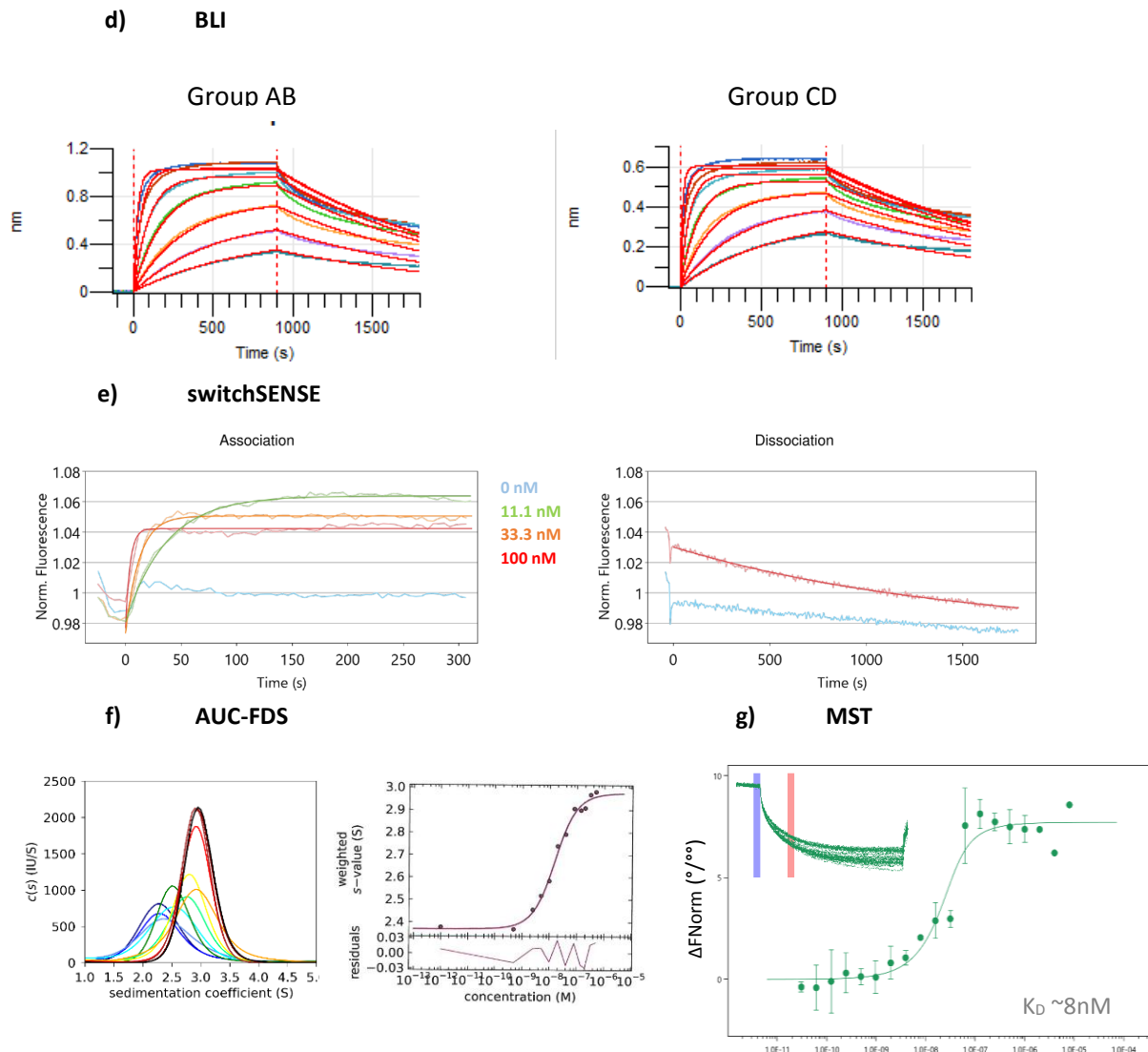
453 (488nm and 505-565 nm respectively). In the MST experiment, labeled A3 was at 35 nM, and  
 454 the highest concentration of Rep17 was 8  $\mu$ M. The titration curves we obtained allowed us to  
 455 measure a  $K_D$  of  $8.0 \pm 4.3$  nM (Fig. 2g).

456 **Figure 2. Biophysical measurements of alphaRep interactions.**



	K (M <sup>-1</sup> )	K <sub>D</sub> (nM)	ΔH (kcal/mol)	α	Δh (kcal/mol)	n
Group A	2.1 · 10 <sup>9</sup>	0.48	12.0	0.015	4.3	0.84
Group B	1.7 · 10 <sup>9</sup>	0.59	10.5	0.036	3.6	0.98
Group C	1.5 · 10 <sup>9</sup>	0.66	12.2	0.041	3.8	0.94
	K <sub>D1</sub> (nM)	ΔH <sub>1</sub> (kcal/mol)	K <sub>D2</sub> (nM)	ΔH <sub>2</sub> (kcal/mol)		
Group A	0.48	12.0	32	16.3		
Group B	0.59	10.5	16	14.1		





457 a) Crystal structure of A3 alphaRep (a-A3, blue) in complex with Rep2 (bA3-2 Ligand concentration ) at 90° view  
 458 (left). b) SEC-MALS analysis. Elution profiles and molar masses of Rep2 in blue, A3 in red, and the complex of A3-  
 459 dimer and two molecules of Rep2 in green. c) PEAQ-ITC data of A3-Rep17 interaction obtained during the training  
 460 for three groups (A, B and C).  $K$ ,  $K_D$ , and  $\Delta H$  were the association constant, the dissociation constant, and the  
 461 interaction enthalpy of either of the two binding sites when the A3 dimer was unoccupied.  $\alpha$  and  $\Delta h$  were the  
 462 cooperative interaction constant and the cooperative interaction enthalpy, which reflect the binding cooperative  
 463 phenomenon (a factor that modulates the binding affinity and a term that modulates the binding enthalpy to the  
 464 second site when there was a site already occupied, respectively).  $n$  was the active (or binding-competent)  
 465 fraction of protein, since the stoichiometry was already included in the model.  $K_{D1}$  and  $\Delta H_1$  were the intrinsic  
 466 site-specific dissociation constant and binding enthalpy for the first binding site ( $K_{D1} = K_D$ ,  $\Delta H_1 = \Delta H$ ).  $K_{D2}$  and  $\Delta H_2$   
 467 were the intrinsic site-specific dissociation constant and binding enthalpy for the second binding site ( $K_{D2} = K_{D1} /$   
 468  $\alpha$ ,  $\Delta H_2 = \Delta H_1 + \Delta h$ ). d) Octet RED96e BLI kinetic analysis of A3-Rep17 interaction in duplicate. Colors code of  
 469 Rep17 concentration: 3.13 nM (teal), 6.25 nM (purple), 12.5 nM (orange), 25 nM (green), 50 nM (cyan), 100 nM  
 470 (red), 200 nM (blue). e) switchSENSE kinetics analysis of A3-Rep17 interaction on dynamic mode, at three Rep17  
 471 concentrations. Normalized association (left) and dissociation (right) data were represented in function of time.  
 472 f) AUC-FDS distribution of sedimentation coefficients (left) with labeled A3 at 5 nM and increasing concentrations  
 473 of Rep17 from 0.5 nM to 0.5  $\mu$ M, obtained with the program SEDFIT (Schuck, 2000) and GUSI (Brautigam, 2015).  
 474 Colors code of Rep17 concentrations: 0  $\mu$ M (navy blue), 0.5 nM (blue), 1 nM (cornflower blue), 2 nM (cyan), 4  
 475 nM (green), 8 nM (springgreen), 16 nM (yellow), 32 nM (orange), 64 nM (red), 128 nM (maroon), 256 nM (brown)  
 476 and 0.5  $\mu$ M (black). AUC-FDS  $K_D$  calculation (right), by fitting with isotherm tool in the program SEDPHAT. g) MST

477 analysis with red fluorophore. Inset, MST traces from relative fluorescence versus time, MST on used was 10 s  
478 (red band).

479 In summary, the students obtained in one week using several complementary approaches,  
480 results that were consistent in stoichiometry, and to a lesser extent, in kinetic rates or affinity  
481 (Table S1). They could observe that the required quantity of sample, ligand or analyte, for each  
482 approach differs significantly. They also observed that some approaches gave highly  
483 complementary information on the system studied (Fig. S2). For example, ITC shows that the  
484 interaction is cooperative when A3 alphaRep is used at the high concentration needed for ITC  
485 (18 $\mu$ M). This cooperativity, linked to the dimeric state of A3, was not observed with  
486 monomeric ligand immobilized on BLI or switchSENSE. They observed higher  $K_D$  values with  
487 AUC-FDS and MST, where one of the partner is labeled, reflecting possible steric hindrance  
488 between the fluorophore and the interaction site.

## 489 **Biophysical measurements of Ku-DNA interactions**

490 We determined the interaction of the heterodimeric full-length protein Ku with four double-  
491 strand DNAs (dsDNAs) of different lengths: 18 bp, 42 bp, 48 bp, and 200 bp (Fig. 3, Fig. S3). Ku  
492 binds DNA through its ring-shaped structure (Fig. 3a). The main observations made during the  
493 training for the Ku-DNA interactions are shown in Fig. 3 and Table S2.

### 494 **Ku-DNA interactions measured by label-free in solution biophysical approaches**

495 For SEC-MALS, we used a Superdex 200 increase 10/300 GL column (*Cytiva*) as above and ran  
496 first a quadruplicate experiment with Ku alone, resulting in an average molar mass of 144.4  
497 kDa (Fig. S3a). We then ran a duplicate experiment with Ku and a 1.2 excess of 42 bp DNA. We  
498 obtained three peaks: the first corresponded to 2 Ku : 1 DNA complex (average Mw of 306.2  
499 kDa), the second corresponded to 1 Ku : 1 DNA (average Mw of 171.0 kDa), and the last  
500 corresponded to the excess of dsDNA alone (average Mw of 26.1 kDa). This suggests that there  
501 is an equilibrium between 2:1 and 1:1 Ku-DNA complexes (Fig. 3b) with stoichiometries  
502 determined by using the protein conjugated method (Loiseau et al.; 2017). The presence of  
503 these two types of complexes could be explain by the use of a non-saturating Ku  
504 concentration.

505 For ITC experiments, we used in our laboratory two different sizes of dsDNA, 18 bp and 42 bp  
506 (Gontier et al., 2021). Due to time limitations students repeated the measurement only with  
507 the 42 bp. We used a VP-ITC instrument which consumes more sample, but was more sensitive  
508 to study these protein-DNA interactions. The heat effects were positive (endothermic  
509 interaction) and small ( $0.2 \mu\text{cal}\cdot\text{sec}^{-1}$ ) (Fig. 3c). Students did two runs, the first at 20  $\mu\text{M}$  and  
510 the second at 40  $\mu\text{M}$  concentration of DNA in the syringe, and in both cases Ku at 4  $\mu\text{M}$  in the  
511 cell. We obtained a mean  $K_D$  at equilibrium of  $3.7 \pm 0.7$  nM with a molar ratio of  $0.34 \pm 0.02$   
512 and a  $\Delta H$  of  $24.4 \pm 2.7$  kcal/mol. The molar ratio was in good agreement with a ratio of 0.5  
513 expected for the interaction of two Ku molecules with a DNA of 42 bp. No evidence for  
514 cooperativity was observed. All the data were analyzed using the Origin software and the new  
515 PEAQ ITC software for comparison.

### 516 **Ku-DNA interactions measured by biophysical approaches with a partner immobilized on a** 517 **surface**

518 Preparatory BLI experiments were performed on an Octet RED384 instrument. **First**, we tried  
519 the strategy that was used for the A3 alphaRep protein, i.e. **capturing the** Ku heterodimer on  
520 Ni-NTA sensors. We did not observe any binding with an 18 bp DNA as analyte (data not  
521 shown). We hypothesized that the DNA binding site may not be accessible when Ku is  
522 immobilized through its His-tag. We therefore tested another strategy, which relied on the  
523 capture of a biotinylated 42 bp DNA on Streptavidin sensors (at 10 nM for 120 s), which were  
524 then incubated with Ku protein as analyte (at concentrations in 200 nM to 1.56 nM range). In  
525 these conditions, we observed an interaction, with an estimated  $K_D$  around 40 nM (data not  
526 shown). We repeated the experiment in duplicate during the training on an Octet RED96e  
527 using an optimized buffer with 1mg/mL BSA and 0.1 % Tween-20, and Ku protein  
528 concentrations ranging from 200 nM to 3 nM. The results of the two runs were consistent,  
529 with apparent kinetic rates,  $k_{ON}$  of  $1.91 \pm 0.02 \cdot 10^{+6} \text{ M}^{-1}\text{s}^{-1}$ ,  $k_{OFF}$  of  $7.17 \pm 0.33 \cdot 10^{-4} \text{ s}^{-1}$ , and a  $K_D$   
530 of  $0.37 \pm 0.01 \text{ nM}$  (Fig. 3d). However, the deviation between the fitted curves and the  
531 experimental data was high, indicating that the interaction mechanism was more complex  
532 than a simple 1:1 binding. For instance, the association and the dissociation processes could  
533 be limited by the diffusion of Ku towards and from the biosensor surface (mass transport  
534 limitation). We therefore analyzed the concentration-dependence of the steady state  
535 responses and measured a  $K_D$  of  $5.2 \pm 0.2 \text{ nM}$  (Fig. S3b). As several curves did not **reach** a  
536 steady state, this  $K_D$  value could however be overestimated and experiments should be  
537 reproduced with longer association times. **Taking everything into account, BLI binding**  
538 **constants were at this stage only qualitative.**

539 Preliminary switchSENSE experiments were done before the training by forming an 80 bp  
540 dsDNA on the sensor. For that, we hybridized an 80-mer DNA with a 32-mer DNA. This DNA  
541 was complementary to the NL48 ssDNA on the chip (Fig. S3c, top, left). We performed kinetic  
542 analyses in duplicate using static mode and Ku protein at 500 nM concentration. We obtained  
543 the following kinetic rates:  $k_{ON}$  of  $9.3 \pm 0.1 \cdot 10^{+4} \text{ M}^{-1}\text{s}^{-1}$ ,  $k_{OFF}$  of  $2.8 \pm 0.1 \cdot 10^{-4} \text{ s}^{-1}$ , and  $K_D$  of  $3.1 \pm$   
544  $0.1 \text{ nM}$  (Fig. S3c, bottom). We monitored the dissociation over a long time (5000 s) to measure  
545 a significant proportion of dissociated Ku molecules. In between, we performed a sizing  
546 measurement. The calculated value ( $D_h = 8.9 \pm 0.3 \text{ nm}$ ) was in good agreement with the one  
547 obtained from the crystal structure of the Ku-DNA complex (PDB: 1JEQ;  $D_h = 8.9 \text{ nm}$ ) (Fig. S3c,  
548 top, right). During the training, we performed experiments in dynamic mode using a 48 bp

549 DNA (without overhang). We used a Ku concentration range from 200 nM to 22.2 nM with 1/3  
550 serial dilution this time. In these conditions, the protein was closer to the fluorophore at the  
551 tip of the 48 bp DNA on the chip. We measured in these conditions a  $k_{\text{ON}}$  of  $2.6 \pm 0.2 \cdot 10^6 \text{ M}^{-1}$   
552  $\text{s}^{-1}$ , a  $k_{\text{OFF}}$  of  $2.3 \pm 0.3 \cdot 10^{-3} \text{ s}^{-1}$ , and a  $K_{\text{D}}$  of  $1.0 \pm 0.2 \text{ nM}$  (Fig. 3e). During the training, students  
553 did not have time to run a sizing experiment.

554 Comparable preliminary data were obtained by SPR using a ProteON XPR36 instrument from  
555 Biorad (Fig. S3d, left), and a Biacore T200 instrument from Cytiva (Fig. S3d, right).

#### 556 **Ku-DNA interactions measured by biophysical approaches with a labeled partner**

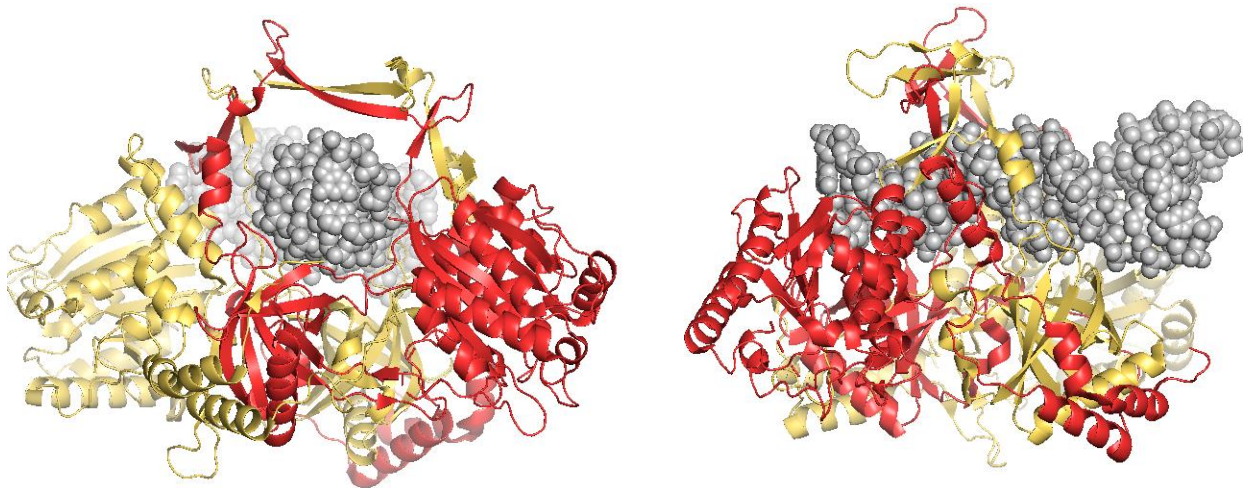
557 For AUC-FDS, we used an 18 bp DNA labeled with fluorescein (FAM) in 5'. We **titrated the DNA**  
558 **(60nM) with Ku (concentration range from 0.5 nM to 0.5  $\mu\text{M}$ )**. The sedimentation profile  
559 showed two species, corresponding to the DNA alone ( $2.15 \pm 0.05 \text{ S}$ ) and to the 1:1 protein-  
560 DNA complex ( $7.25 \pm 0.15 \text{ S}$ ), and we were able to determine the  $K_{\text{D}}$  at equilibrium ( $12.9 \pm 3.2$   
561 nM) (Fig. 3f).

562 For MST, we used the **same 18 bp labeled DNA** as for AUC-FDS, but at 10 nM; and **titrated it**  
563 **with Ku (concentration range from 2  $\mu\text{M}$ )**. Training participants performed four runs in total,  
564 in duplicates or triplicate (Fig. 3g). We could see some variability in the curves that might  
565 originate from the pipetting of the different students. All curves were fitted globally resulting  
566 in a  $K_{\text{D}}$  of 11.9 nM.

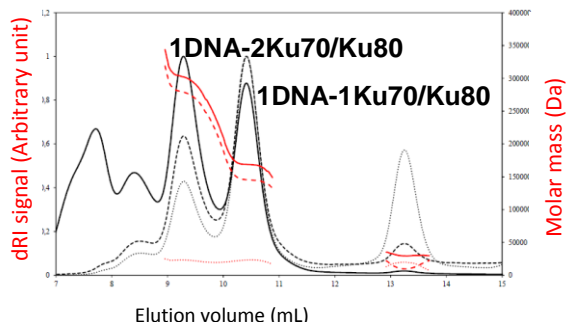
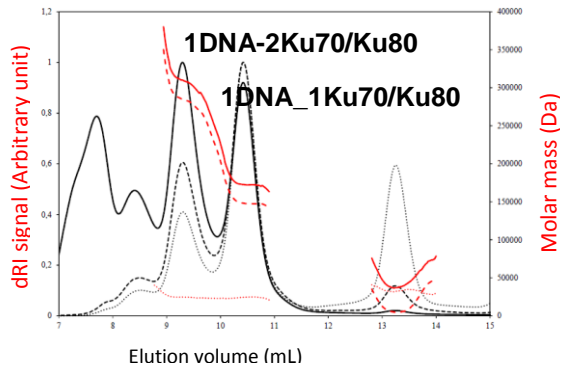
567 Comparable data were obtained **in parallel of the training** with a novel technology,  
568 microfluidic diffusional sizing (MDS) in collaboration with Fluidic Analytics (Fig. S3e). The  
569 Fluidity One-W instrument measures the rate of diffusion of macromolecules under steady  
570 state laminar flow in a microfluidic chip. In a diffusion chamber with two parallel streams, the  
571 migration of a labeled partner depends on its size. At the end, the streams are re-split and the  
572 **fluorescence** ratio between the **two allows to calculate  $R_{\text{h}}$** . **The variation of  $R_{\text{h}}$  observed when**  
573 **titrating** the labeled partner **by the unlabeled one allows to generate** a binding curve and to  
574 calculate a  $K_{\text{D}}$  value.

575 **Figure 3. Biophysical measurements of Ku-DNA interactions.**

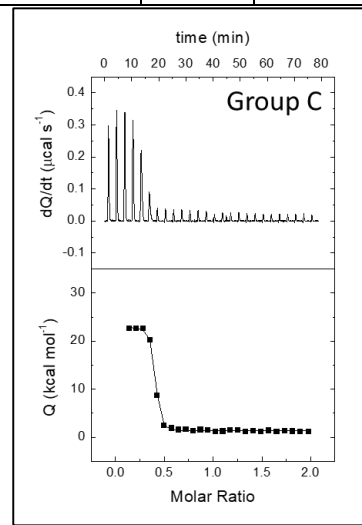
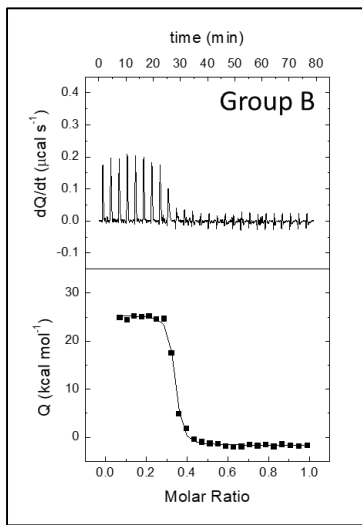
a) **Ku-DNA**



b) **SEC-MALS**



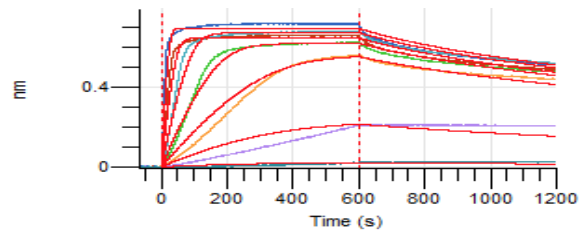
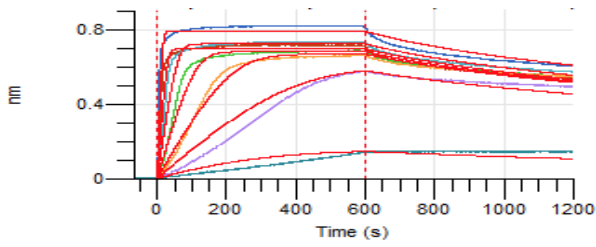
c) ITC



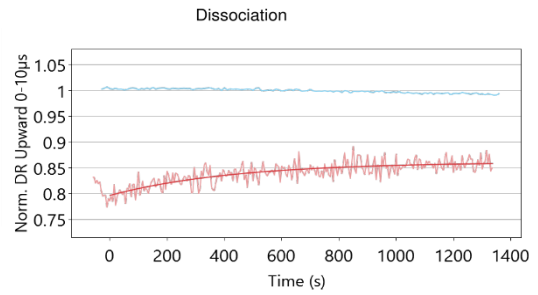
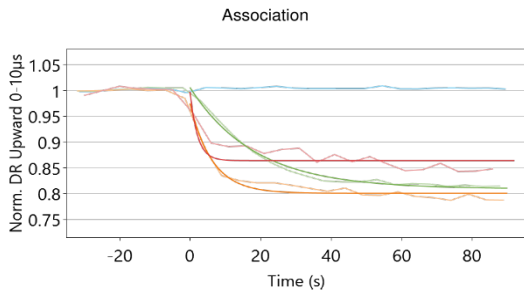
		Peak 1	Peak 2	Peak 3
Group C	Protein molar mass	279.1	145.7	9.6
	DNA molar mass	23.3	23.3	19.9
	Total molar mass	302.3	169.0	29.6
Group D	Protein molar mass	285.3	148.4	4.8
	DNA molar mass	24.7	24.6	32.3
	Total molar mass	310.1	172.9	37.1

	K (M <sup>-1</sup> )	K <sub>b</sub> (nM)	ΔH (kcal/mol)	n
Group B	3.4 10 <sup>8</sup>	3.0	27.1	0.32
Group C	2.3 10 <sup>9</sup>	4.3	21.7	0.37

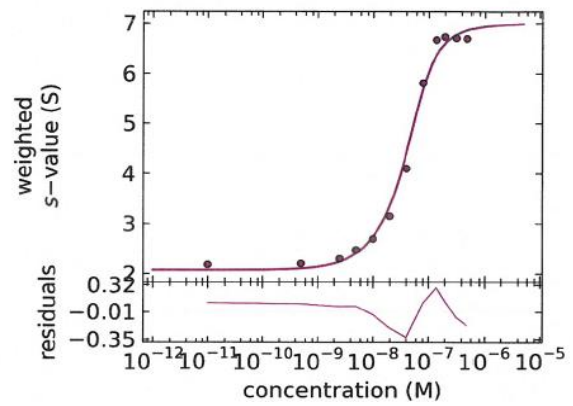
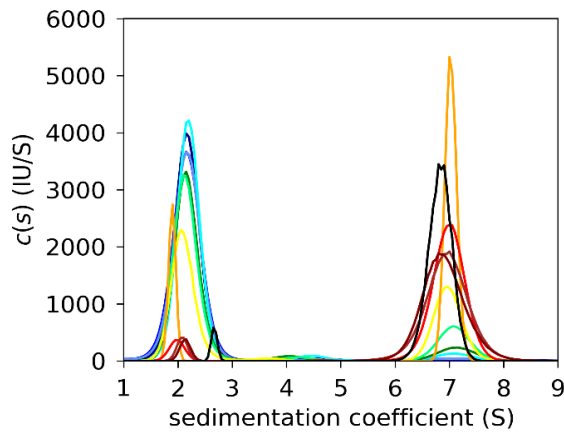
d) BLI: Group AB and Group CD



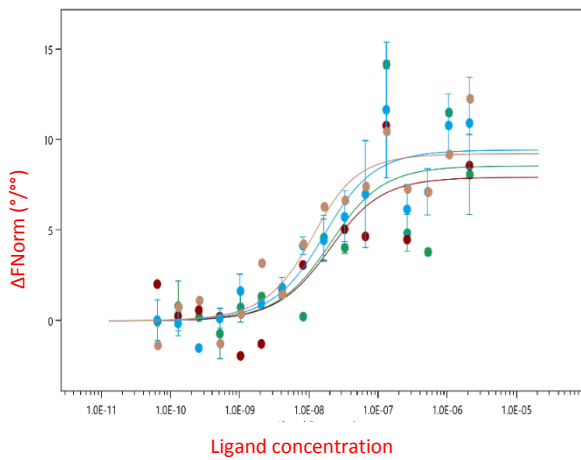
e) **switchSENSE**



f) **AUC-FDS**



g) **MST**



GROUPS	Excitation Power	$K_D$	$K_D$ Confidence	Std. Error of Regression	Signal to Noise
A1-B1	60	1.53 $10^{-08}$	1.86 $10^{-08}$	2.77	3.33
A2	40	1.49 $10^{-08}$	1.73 $10^{-08}$	2.01	4.33
B2-C2-D2	100	1.14 $10^{-08}$	7.26 $10^{-09}$	1.61	6.31
D1	100	6.15 $10^{-09}$	4.3 $10^{-09}$	1.56	6.37

576 a) Crystal structure of the Ku- DNA complex (PDB: 1JEQ). View down the DNA helix (left) and side view (right),  
 577 with Ku70 colored in red, Ku80 in yellow cartoons and DNA in grey spheres. b) SEC-MALS elution profiles and  
 578 molar masses of Ku-DNA complex in duplicate. Representation of elution volumes in mL and molar mass in g/mol.  
 579 c) VP-ITC data in duplicate. d) Octet RED96e BLI kinetics data in duplicate. Colors code of Ku concentration: 3.13



580 nM (teal), 6.25 nM (purple), 12.5 nM (orange), 25 nM (green), 50 nM (cyan), 100 nM (red), 200 nM (blue). e)  
581 switchSENSE dynamic mode data for three Ku concentrations. F) AUC-FDS measurement of Ku-DNA interaction.  
582 AUC-FDS distribution of sedimentation coefficients (left) with labeled DNA at 60 nM and increasing  
583 concentrations of Ku from 0.5 nM to 0.5  $\mu$ M, obtained with the program SEDFIT (Schuck, 2000) and GUSI  
584 (Brautigam, 2015). Colors code of Ku concentration: 0  $\mu$ M (navy blue), 0.5 nM (blue), 1 nM (cornflower blue), 2  
585 nM (cyan), 4 nM (green), 8 nM (springgreen), 16 nM (yellow), 32 nM (orange), 64 nM (red), 128 nM (maroon),  
586 256 nM (brown) and 0.5  $\mu$ M (black). AUC-FDS  $K_D$  calculation by fitting with isotherm tool in the program SEDPHAT  
587 (right). g) MST analysis. Representation of ligand concentration against  $\Delta F_{\text{Norm}} \text{ } ^\circ/\text{ } ^\circ$  (left). Superposition of  
588 several run in the similar conditions, but different excitation power and students. Summary of MST data (right).

589 In summary, for the Ku-DNA project, the students also obtained consistent results of  
590 stoichiometry, kinetic rates and affinity (Fig. 3, Table S2). As for the alphaRep project, the  
591 quantity of sample required varies between the different approaches. ITC, SEC-MALS and AUC-  
592 FDS were very efficient for measuring the stoichiometry of Ku on DNA with one Ku molecule  
593 bound every 18-21bp DNA. They observed that measurements on surfaces, either by BLI or  
594 switchSENSE, were successful only when the DNA was immobilized. They could observe that  
595 switchSENSE allows a quite accurate estimation of the Ku size (Fig. S3d, bottom).  
596 Oligonucleotides with a fluorescent probe in 5' or 3' are inexpensive and allow to follow the  
597 interaction between a fluorescent DNA and a protein quite easily by MST or AUC-FDS, without  
598 major steric hindrance between the fluorescent probe and the interaction sites.

## 599 Discussion

600 The study presented here compares six different *in vitro* biophysical approaches to  
601 characterize the architecture (in terms of size or stoichiometry) and binding parameters ( $k_{\text{ON}}$ ,  
602  $k_{\text{OFF}}$ ,  $K_D$ ) of two different types of complexes (protein-protein and protein-DNA). It was  
603 designed to provide a rather complete overview of six different techniques in a short period.  
604 During a week, 20 participants performed this study in the context of the MoSBio Training  
605 School (ARBRE-MOBIEU COST Action). During the first day of the training school, experts of  
606 each field presented the projects, the theory and examples of applications for the three  
607 classical techniques (AUC, SEC-MALS, ITC), and the three more recent ones (MST, BLI,  
608 switchSENSE). An additional presentation about, sample quality control, a relevant subject  
609 regarding reproducibility of experimental measurements, completed the first day of the  
610 training (Raynal et al., 2014). The remainder of the week was dedicated fulltime to practical  
611 sessions where the participants could perform experiments on instruments, analyze results  
612 and discuss with experts. All six approaches, except AUC, and SEC-MALS for the alphaRep  
613 interaction, were successfully used during the training school to study two important types of  
614 macromolecular interactions (alphaReps and Ku-DNA). The training allowed the students,

615 first, to compare the quantity of material consumed for each technique, and, second, to  
616 understand the parameters that can be measured by each of them (Fig. 4). This first edition of  
617 the MoSBio Training School was positively assessed both **by trainers and trainees**. It was a  
618 unique opportunity to compare advantages and limitations of this large ensemble of  
619 techniques. A new edition will be scheduled soon. The participants were able to use all  
620 instruments quite easily by themselves, with the exception of AUC, which requires a little more  
621 expertise and longer run times. Finally, this study showed that a nanomolar range affinity **can**  
622 **be easily** assessed with most tested techniques. **The students could observe that the  $K_D$  vary**  
623 **significantly with the technique used, though the  $K_D$  measured were all in the nanomolar**  
624 **range. The maximal and minimal values of  $K_D$  measured between the alphaRep and the**  
625 **different techniques differ by a factor 21. The extreme  $K_D$  differ by a factor 13 for the  $K_D$**   
626 **measured between Ku and DNA. We observed that in these two systems the presence of a**  
627 **label on one partner, in MST and AUC-FDS approaches, comes with a higher  $K_D$  than without**  
628 **label. During the training school, the students could observe the variability on the  $K_D$  values**  
629 **measured in the different groups or with the different approaches (see Fig. 2 and Fig. 3). The**  
630 **training was also an opportunity to discuss on the techniques that are more favorable to**  
631 **measure lower (pM) or higher ( $\mu$ M)  $K_D$  values.**

632 The results presented here for the alphaRep-proteins and Ku-DNA complexes highlight the  
633 advantages and drawbacks of each approach. The **quantity** of required **sample** was not limited  
634 in our case, but the participants clearly observed that the amounts of protein and DNA used  
635 for each approach are very different (Fig. 4). AUC, SEC-MALS and ITC are the most sample-  
636 consuming techniques. **The ITC200 and PEAQ-ITC instruments** were an important progress for  
637 ITC in this regard, but we observed that for interactions with very weak heat exchange like Ku-  
638 DNA, the more sensitive, but more sample-consuming VP-ITC was still needed to obtain good  
639 results. Surface methods like BLI and switchSENSE require only small amounts of the  
640 immobilized protein. For the study of high affinity systems (in the nM range) like those  
641 characterized here, the consumption of analyte is small too. For weaker affinities (higher  $K_D$  in  
642 the  $\mu$ M range) the **required quantity** of analyte will however rise significantly to cover  
643 concentrations from 1/10 of the  $K_D$  to 10 times the  $K_D$ . The MST technique consumes very  
644 small volumes of material once the labeling step is successfully achieved. Anyway, the time  
645 and sample consumption for a given technique must be evaluated not just for a single

646 successful experiment, but must also **take into account** the experimental design and  
647 optimization stage.

648 **Figure 4. Comparison of results obtained for the alphaReps and the Ku-DNA interaction with**  
649 **the six approaches.**

Alpha Reps	SEC- MALS	200 µg A3, 200 µg Rep2	ITC	80 µg A3, 130 µg Rep17
		Molar mass Stoichiometry: (A3-dimer): 2 Rep2		Biphasic curve Cooperativity (A3 dimer) $K_{D1}$ , $K_{D2}$ , $\Delta H$ , $-\Delta\Delta S$
	BLI	8 µg A3, 2 µg Rep17	Switch- SENSE	150 ng A3, 2-20 µg Rep17
		A3-monomer $K_D$ , $K_{ON}$ , $K_{OFF}$		A3-monomer Size ( $D_h$ ) $K_D$ , $K_{ON}$ , $K_{OFF}$
	AUC- FDS	1 µg A3, 8 µg Rep17	MST	620 ng A3, 6 µg Rep17 (One labeling failed)
		Sedimentation coefficient Stoichiometry $K_D$		$K_D$
Ku- DNA	SEC- MALS	40µg of DNA, 200 µg Ku	ITC	990 µg Ku, 320 µg DNA
		Molar mass Stoichiometry: 1 Ku /18-21 bp		Biphasic curve Stoichiometry: 1 Ku /18-21bp $K_D$ , $\Delta H$ , $-\Delta\Delta S$
	BLI	400 ng DNA, 14 µg Ku (Failed with Ku immobilized)	Switch- SENSE	150 ng DNA, 2-20 µg Ku (Ligand is the nanolever)
		$K_D$ , $K_{ON}$ , $K_{OFF}$		Size ( $D_h$ ) $K_D$ , $K_{ON}$ , $K_{OFF}$
	AUC- FDS	3.5 µg DNA, 80 µg Ku (DNA easily labeled)	MST	50 µg DNA, 12 µg Ku (DNA easily labeled)
		Sedimentation coefficient Stoichiometry $K_D$		$K_D$

650 The experiments performed by the students on the two systems of study with the six approaches were  
651 successful. The sample quantities used for the different approaches are indicate, as well as the parameters that  
652 can be deduced from the different approaches.

653 All experimental approaches used in this study can provide  $K_D$  values of the interaction.

654 Interestingly, they also provide additional information that may orient the users to one or the  
655 other according to the main questions raised in their specific projects. In the two examples we  
656 studied, the stoichiometry issue is an important point, since the A3 alphaRep is a dimer when  
657 its concentration is in the µM range, while it is a monomer in the nM range. According to this  
658 oligomeric state, we can observe some cooperative binding of its partner, which influences  
659 the binding parameters. In this regard, ITC, SEC-MALS and AUC allow characterizing the ratio  
660 between the dimeric alphaRep target and its binders or between Ku and DNA of increasing

661 lengths, and confirm the information provided by **the** crystal structures **of the complexes**. The  
662 sizing measurement in switchSENSE can also provide some information regarding the  
663 stoichiometry **or** conformation.

664 Kinetic parameters are important data to estimate the half-time of a complex, which is an  
665 **important** interaction parameter. BLI and switchSENSE (as well as SPR) proved to be efficient  
666 in this study to determine  $k_{ON}$  and  $k_{OFF}$ . We observed that the switchSENSE allows to  
667 characterize tight interactions with small  $k_{OFF}$  values ( $k_{OFF}$  about  $10^{-4}$  s) by monitoring the  
668 dissociation over a long time period (5000 s). Thermodynamic parameters (enthalpy, entropy  
669 and Gibbs free energy) also provide key information about macromolecular interactions,  
670 notably about the polar/apolar nature of the interface between a protein and its ligand. The  
671 Ku-DNA thermograms for example indicate that the interaction is entropically driven with a  
672 positive and unfavorable enthalpy probably linked to desolvation of the DNA and Ku surface  
673 upon binding.

674 The choice of one technique over another depends mainly on the questions one wants to  
675 answer. Some practical issues can also guide the choice of the method, such as the quantity  
676 and solubility of protein and ligands available, the possibility to immobilize or to label one of  
677 the interaction partners, and the time available. The presence of an instrument and experts  
678 in **house** or nearby the user is another criterion. Noteworthy, all these approaches are  
679 available in many research institutions in Europe, especially in those **engaged** in Structural  
680 Biology studies. If needed, these approaches are also available through **research**  
681 infrastructures, like **MOSBRI and** Instruct in Europe or FRISBI in France. Finally, there are  
682 research networks, like ARBRE-MOBIEU, allowing **to foster** fruitful exchanges with experts in  
683 the different fields. The MoSBio training school over a week with 20 students proved to be a  
684 very rich occasion for such discussions.

685 Studying macromolecular interactions *in vitro* **is** extremely complementary to *in cellulo*  
686 analyses. Interactions observed in cells or in cellular extracts often need **further** *in vitro*  
687 **characterization of** target engagement. Training **schools**, as the one described in this article,  
688 are central to further disseminate the importance of biophysical studies in **cell** biology  
689 laboratories. This training school **contributed** to initiate students from various fields **to**  
690 biophysical approaches and **showed** in a short period the added-value of quantitative  
691 measurements of protein interactions.

692 By mutagenesis, Nuclear Magnetic Resonance (NMR) shift or Hydrogen-Deuterium Exchange  
693 Mass Spectrometry (HDX-MS), one can determine the residues involved in the interaction, and  
694 structural analysis can yield information about their topology. One can play with the  
695 experimental conditions (pH, ionic strength, temperature) to expand the knowledge about the  
696 nature of the interaction under study (electrostatic and/or hydrophobic, exo- or endothermic,  
697 presence/absence of conformational changes, coupling with additional binding equilibria).  
698 Furthermore, pharmacological studies require this kind of biophysical approaches. Finally,  
699 molecular scale biophysics data can also be used to implement *in silico* simulations to predict  
700 other interactions in basic research. A second edition of this Training School will be held as  
701 soon as possible. The extremely positive feedback from the students, as well as from the  
702 academic experts and industrials participating in this school, highlights the need for a better  
703 understanding of the theoretical bases of such a panel of biophysical methods coupled to  
704 hands-on experimental practice.

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800

801 **Declarations**

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807 commercial interest

808 **Ethics approval** Not applicable.

809 **Consent to participate** The authors consent to participate to this project.

810 **Consent for publication** The authors consent to publish the work reported in this paper.

811 **Availability of data and material** Data can be obtained by requesting the corresponding  
812 author.

813 **Code availability** Not applicable.

814 **Authors' contributions** PFV, PE, SU, CE, AVC, AR, JBC authors contributed to the study  
815 conception and design. Material preparation, data collection and analysis were performed by  
816 CV, MAN, SU, PE, AVC, DS, GB, PS, CQ, CE, AR. The first draft of the manuscript was written by  
817 PFV and all authors commented on previous versions of the manuscript. All authors read and  
818 approved the final manuscript.

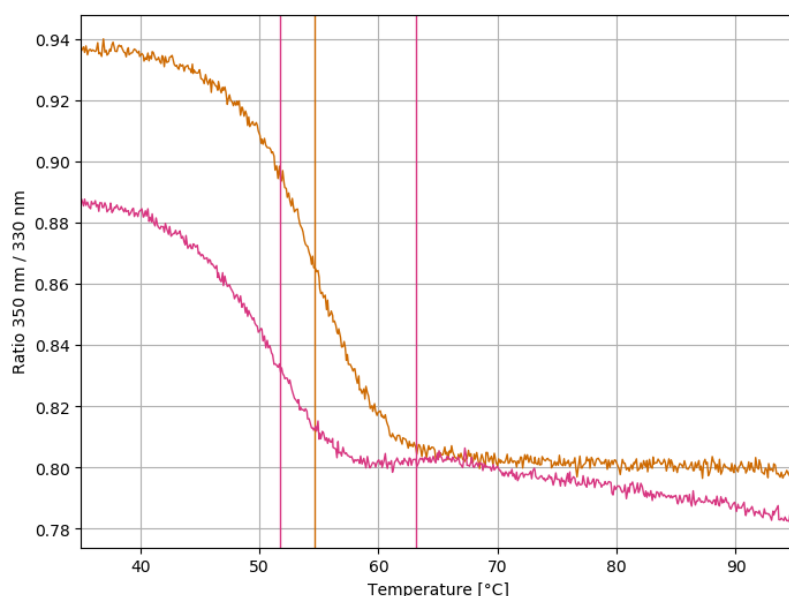
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831 (<https://www.pluginlabs-universiteparisclay.fr/fr/results/keywords/PIM>), while some  
832 others were performed in Institut Pasteur, PFBMI platform.

833 **Supplementary material**

834 **Figures**

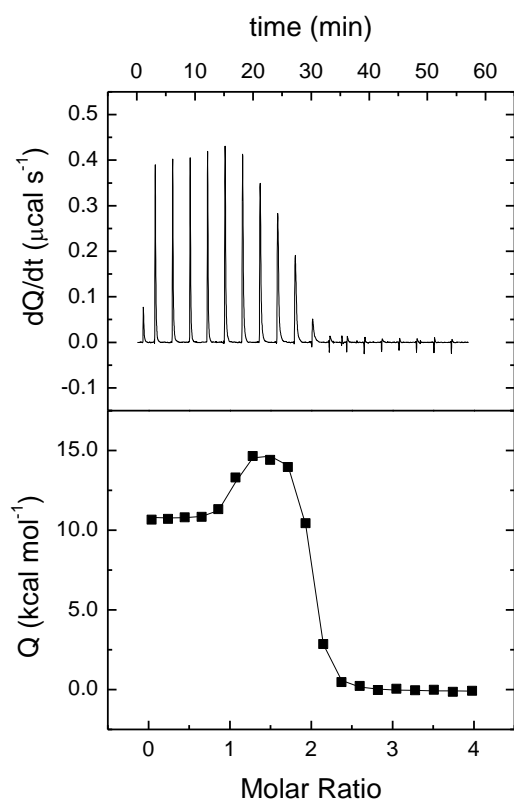
835 **Figure S1. Unfolding profile plot of Ku70/Ku80.**



836 Quality and stability of samples are crucial to obtain accurate biophysical data. For all the biophysical studies  
837 shown in this manuscript, we used a full-length version of Ku (called KuFL), but for structural studies we used a  
838 shorter version where the C-terminus of each monomer is deleted (called KuCC). We took advantage of the new  
839 technology of *NanoTemper*, the Tycho NT.6 instrument, present during the training, to compare the thermal  
840 stability of these two versions of Ku protein by a fast measurement. Fluorescence intensity is recorded at 330 nm  
841 and 350 nm (emission profile of the Tryptophan residues). The brightness ratio 350 nm / 330 nm plotted against  
842 the temperature is called the unfolding profile plot and inflexion temperatures can be derived representing  
843 unfolding events. Tryptophan fluorescence of KuFL (orange) and KuCC (pink) were follow during a ramp of  
844 temperature of 35-95°C. KuFL showed a higher temperature of unfolding than KuCC (vertical bars). Thus, KuFL  
845 appears to be more stable than KuCC by a few degrees.

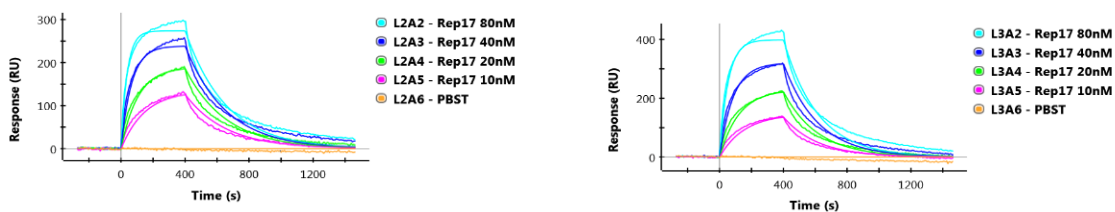
846 **Figure S2. Additional biophysical measurements of alphaRep interactions.**

**a) ITC**

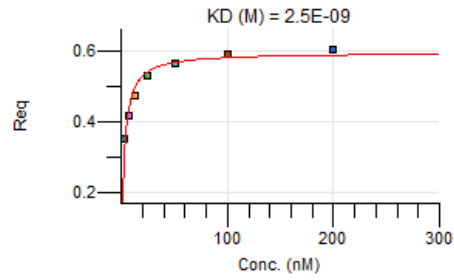
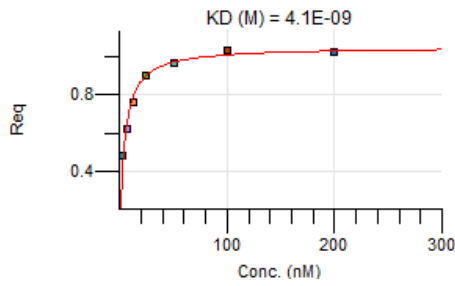


K ( $\text{M}^{-1}$ )	$K_D$ (nM)	$\Delta H$ (kcal/mol)	$\alpha$	$\Delta h$ (kcal/mol)	n
$1.4 \cdot 10^9$	0.71	10.9	0.024	4.4	0.95

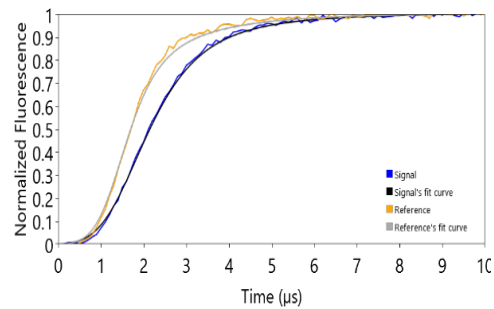
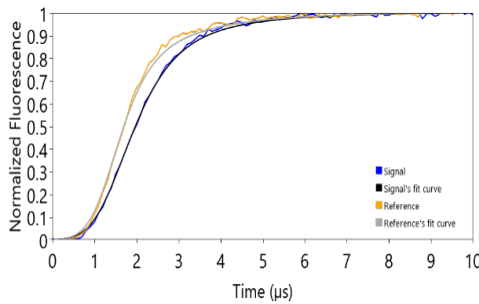
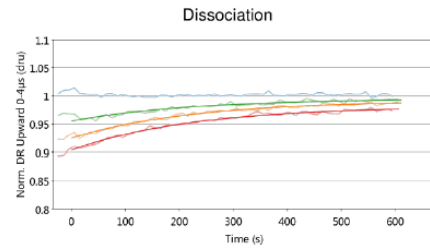
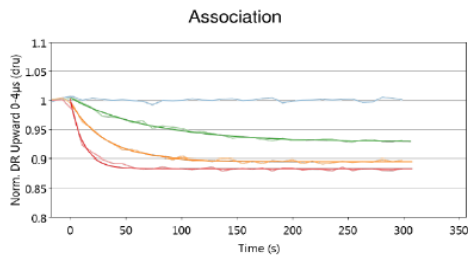
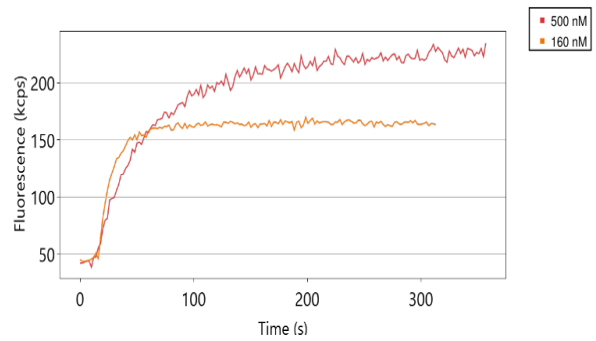
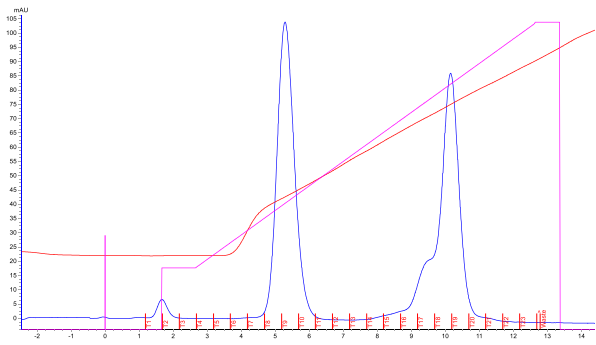
**b) SPR**



**c) BLI**



**d) switchSENSE**



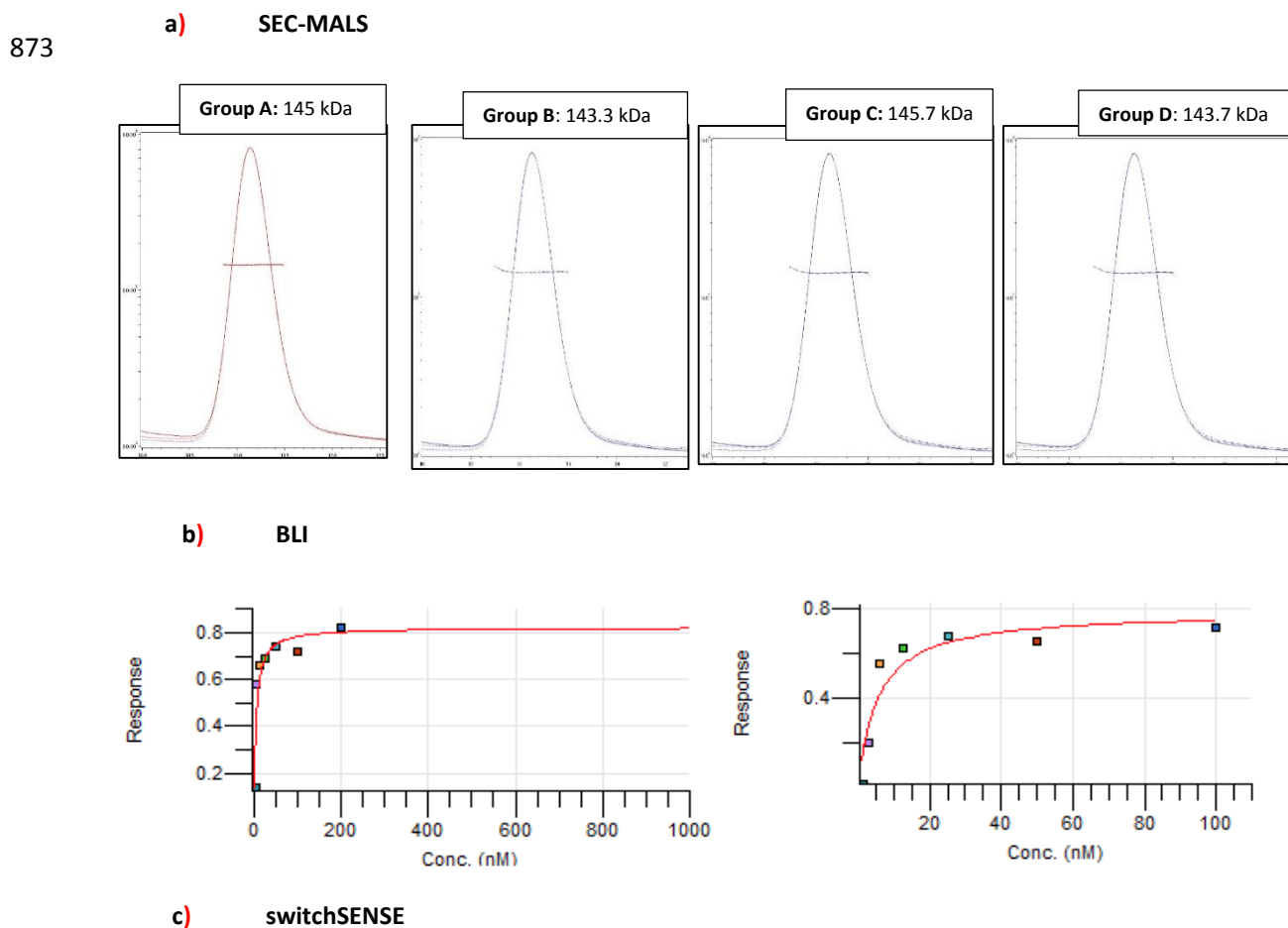
$D_h^{ave} = 3.02 \pm 0.05 \text{ nm} \text{ (n=8)}$

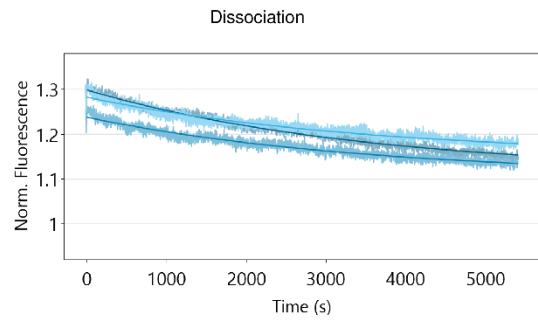
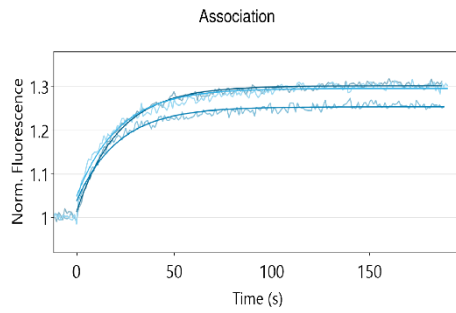
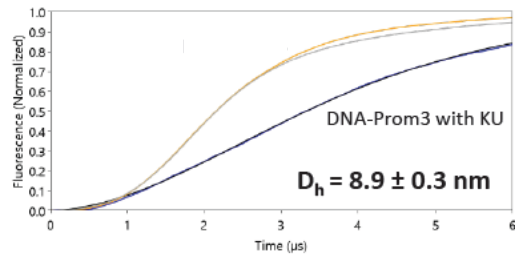
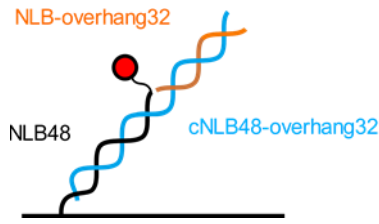
$D_h^{ave} = 3.82 \pm 0.09 \text{ nm} \text{ (n=4)}$

847 a) PEAQ-ITC data obtained before the training in our laboratory.  $K$ ,  $K_D$ , and  $\Delta H$  were the association constant, the  
 848 dissociation constant, and the interaction enthalpy of either of the two binding sites when the dimer was  
 849 unoccupied.  $\alpha$  and  $\Delta h$  were the cooperative interaction constant and the cooperative interaction enthalpy, which  
 850 modulate the binding cooperative phenomenon (a factor that modulates the binding affinity and a term that  
 851 modulates the binding enthalpy to the second site when there was a site already occupied, respectively).  $n$  was  
 852 the active (or binding-competent) fraction of protein, since the stoichiometry was already included in the model.  
 853  $K_{D1}$  and  $\Delta H_1$  were the intrinsic site-specific dissociation constant and binding enthalpy for the first binding site

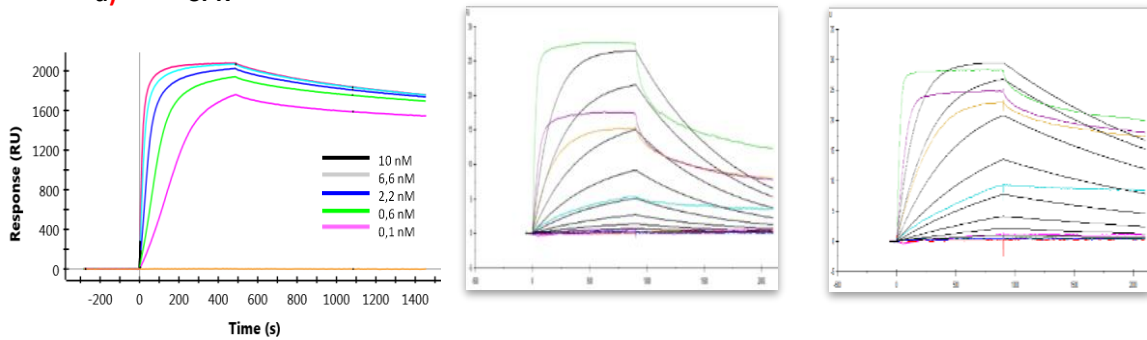
854 ( $K_{D1} = K_D$ ,  $\Delta H_1 = \Delta H$ ).  $K_{D2}$  and  $\Delta H_2$  were the intrinsic site-specific dissociation constant and binding enthalpy for  
 855 the second binding site ( $K_{D2} = K_{D1} / \alpha$ ,  $\Delta H_2 = \Delta H_1 + \Delta h$ ). b) Sensograms of alphaRep A3-Rep17 interaction measured  
 856 in duplicate on a ProteON XPR36 instrument from *Biorad*. For this experiment, association and dissociation times  
 857 were 400 s and 1000 s, respectively with a flow rate of 50  $\mu\text{L}/\text{min}$  in phosphate buffered saline with 0.1 % Tween-  
 858 20 (PBST) using His-Tag capturing (HTG) chip to immobilized A3 at 6.25 or 12.5  $\mu\text{g}/\text{mL}$  during 80 s (inducing 60  
 859 RU and 95 RU, respectively). Rep17 ranges from 10 to 80 nM concentration. A  $k_{\text{ON}}$  of  $2.8 \cdot 10^{+5} \text{ M}^{-1}\text{s}^{-1}$ , a  $k_{\text{OFF}}$  of  $3.9$   
 860  $10^{-3} \text{ s}^{-1}$  and a  $K_D$  of 13.8 nM were measured in the first run, and a  $k_{\text{ON}}$  of  $1.8 \cdot 10^{+5} \text{ M}^{-1}\text{s}^{-1}$ , a  $k_{\text{OFF}}$  of  $4.6 \cdot 10^{-3} \text{ s}^{-1}$  and a  
 861  $K_D$  of 25.9 nM in the second one. c) Octet RED96e BLI steady-state analysis in duplicate. Colors code of Rep17  
 862 concentrations: 3.13 nM (teal), 6.25 nM (purple), 12.5 nM (orange), 25 nM (green), 50 nM (cyan), 100 nM (red),  
 863 200 nM (blue). d) Sample preparation for switchSENSE experiments. Anion-exchange chromatogram of the A3  
 864 cross-linked protein (up, left). The cross-linked protein was the shoulder of the final peak that correspond to the  
 865 free DNA. Hybridization of the cross-linked ssDNA-A3 on the chip, which carries a red fluorescent probe (up,  
 866 right). Red profile corresponds to the control (free cNLB48 without protein) and the orange one to the cross-  
 867 linked cNLB48-A3 conjugate. switchSENSE dynamic mode data of A3-Rep2 at three concentrations (middle, left).  
 868 Normalized association (left) and dissociation (right) data were represented in function of time. switchSENSE  
 869 sizing measurement of A3 hydrodynamic diameter ( $D_h$ ) (bottom, left), and of the A3-Rep17 complex (bottom,  
 870 right). Measured signal curve is in blue (conjugated DNA), the associated fitted curve in blank, reference signal  
 871 (cNLB48) is in orange, and the associated fitted curve in grey.

872 **Figure S3. Additional biophysical measurements of Ku-DNA interactions.**

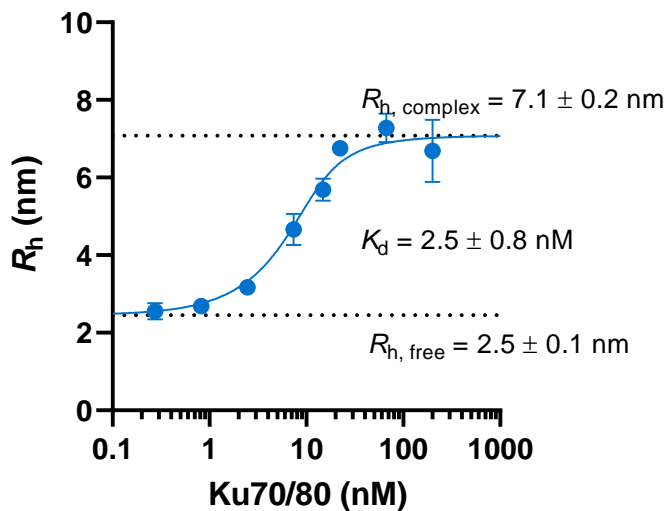




**d) SPR**



**e) MDS**



874 a) SEC-MALS elution profiles and molar masses of Ku alone in quadruple. b) Octet RED96e BLI steady-state data  
 875 in duplicate. Colors code of Ku concentration: 3.13 nM (teal), 6.25 nM (purple), 12.5 nM (orange), 25 nM (green),  
 876 50 nM (cyan), 100 nM (red), 200 nM (blue). c) switchSENSE measurement set up (top, left). By hybridization of  
 877 complementary DNA carrying the target sequence as overhang, the surface is functionalized with the sequence

878 of interest. switchSENSE static mode data of 1 Ku concentration in duplicate (bottom). switchSENSE sizing data  
879 (top, right). Reference (bare DNA) is depicted in yellow. DNA-protein-complex is depicted in blue. d) SPR  
880 sensograms by ProteON XPR36 of Ku-200 bp dsDNA interaction measured (right). A 200 bp biotinylated DNA was  
881 immobilized on a Streptavidin (NLC) chip. Ku70/Ku80 ranged from 0.1 to 10 nM concentrations, one heterodimer  
882 may induce 200 RU. A  $k_{ON}$  of  $7.8 \pm 4 \cdot 10^{+6} \text{ M}^{-1}\text{s}^{-1}$ , a  $k_{OFF}$  of  $1.5 \pm 0.8 \cdot 10^{-4} \text{ s}^{-1}$  and a  $K_D$  of  $7.3 \pm 3 \text{ nM}$  were measured.  
883 This experiment showed the threading of Ku on DNA, approximately 10 heterodimers bound to this 200 bp DNA.  
884 SPR sensograms by BIAcore T200 of Ku-42 bp dsDNA (middle) and, Ku-60 bp dsDNA (left) interaction measured  
885 by biotinylated DNA immobilized in a serie S sensor chip SA at 10 nM concentration. Same buffer as in  
886 switchSENSE experiment adding 0.2 mg/mL of BSA, flow rates of 20 to 100  $\mu\text{L}/\text{min}$ , and Ku at 5 nM as higher  
887 concentration for 900 s of association and dissociation. A  $k_{ON}$  of  $1.0 \cdot 10^{+6} \text{ M}^{-1}\text{s}^{-1}$ , a  $k_{OFF}$  of  $11.7 \cdot 10^{-4} \text{ s}^{-1}$  and a  $K_D$  of  
888 1.2 nM ( $R_{max}$  of 32.9 RU) were measured for the 42 bp DNA and, a  $k_{ON}$  of  $1.4 \cdot 10^{+6} \text{ M}^{-1}\text{s}^{-1}$ , a  $k_{OFF}$  of  $5.0 \cdot 10^{-4} \text{ s}^{-1}$  and  
889 a  $K_D$  of 35.4 nM ( $R_{max}$  of 31.6 RU) for the 60 bp DNA. Colors code of Ku concentrations: 5 nM (light green), 2.5 nM  
890 (purple), 1.25 nM (orange), 0.625 nM (cyan), 0.3125 nM (pink), 0.156 nM (dark green), 0.078 nM (blue) and 0.039  
891 nM (red). The differences observed in the results depend mainly on the setup strategy of the experiment.  
892 Nevertheless, open questions remain to answers: the purity of dsDNA and the stability of the protein will affect  
893 the results, but there is also the possibility of different ways of DNA fixation by Ku. e) In collaboration with *Fluidic*  
894 *Analytcs*, we collected preliminary data to test their new instrument, Fluidity One-W with the Ku-DNA  
895 interaction. This is a novel technique in solution based in diffusional sizing of a complex in a microfluidic system.  
896 For this experiment we used the 18 bp dsDNA-FAM as before (AUC, MST) at 10 nM concentration, and 1/3  
897 dilution of Ku70/Ku80 from 200 to 0.09 nM concentration. We were able to measure  $R_h$  (DNA alone, complex)  
898 and  $K_D$  values.

## 899 Tables

900 **Table S1. Summary of the results for the interaction between alphaRep proteins using**  
901 **different techniques.**

	AUC-FDS	SEC-MALS*	ITC		MST	switchSENSE		BLI		SPR*
His-A3	Blue-NHS	Dimer	Monomer /high affinity	Dimer /low affinity	Red-His	Rep2*	Rep17	Monomer		Monomer
Ratio	1:1	1:1	1:1	1:1	N/A	1:1	1:1	N/A		N/A
$K_D$ (nM)	$13.4 \pm 1.8$	N/A	$0.6 \pm 0.1$	$21.3 \pm 7.5$	8.0	$0.29 \pm 0.01$	$0.98 \pm 0.16$	$3.2 \pm 1.1$	$3.3 \pm 0.8$	$3.97 \pm 1.38$
$k_{ON}$ ( $\text{M}^{-1}\text{s}^{-1}$ ) $k_{OFF}$ ( $\text{s}^{-1}$ )	N/A	N/A	N/A	N/A	N/A	$2.4 \pm 0.1 \cdot 10^{+6}$ $6.9 \pm 0.2 \cdot 10^{-4}$	$2.4 \pm 0.2 \cdot 10^{+6}$ $2.3 \pm 0.3 \cdot 10^{-3}$	$2.6 \pm 0.7 \cdot 10^{+5}$ $7.6 \pm 0.7 \cdot 10^{-4}$	Steady-state	$2.31 \pm 1.79 \cdot 10^{+5}$ $4.28 \pm 0.36 \cdot 10^{-3}$
Other information	S (A3) = $2.2 \pm 0.1$ S (A3/Rep17) = $3.0 \pm 0.1$	Mw (Rep2) = 11.6 kDa Mw (A3) = 44.8 kDa $R_h$ (A3) dimer = 3nm Mw (A3/Rep2) = 65.5 kDa $R_h$ (A3/Rep2) = 3.8 nm	$\Delta H = 11.6 \pm 0.6$ kcal/mol	$\Delta H = 15.5 \pm 1.0$ kcal/mol	N/A	$D_h$ (A3) monomer = 3 nm $D_h$ (A3-Rep2) = 3.8 nm	N/A	N/A	N/A	N/A



902 In the last column in grey, previous SPR data obtained by P. Minard's team. \*Rep2

903 **Table S2. Summary of the results for the interaction Ku-DNA using the different**  
 904 **techniques.**

	AUC-FDS	ITC		SEC-MALS	BLI		MST	switch SENSE		SPR	MDS
DNA(bp)	18*	18	42	42	42**		18*	48	80	200**	18*
Ratio Ku vs DNA	1:1	1:1	2:1	2:1, 1:1	N/A		N/A	1:1		20:1	1:1
K <sub>D</sub> (nM)	12.9 ± 3.2	0.71	3.7 ± 0.7	N/A	0.4 ± 0.1	5.2 ± 0.2	11.9	1.0 ± 0.2	3.1 ± 0.1	7.3 ± 3.0	2.5 ± 0.8
K <sub>ON</sub> (M <sup>-1</sup> s <sup>-1</sup> ) K <sub>OFF</sub> (s <sup>-1</sup> )	N/A	N/A	N/A	N/A	1.9 ± 0.1 10 <sup>+6</sup> 7.2 ± 0.3 10 <sup>-4</sup>	N/A	N/A	2.6 ± 0.2 10 <sup>+6</sup> 2.3 ± 0.3 10 <sup>-3</sup>	9.3 ± 0.1 10 <sup>+4</sup> 2.8 ± 0.1 10 <sup>-4</sup>	7.8 ± 4.0 10 <sup>+6</sup> 1.5 ± 0.8 10 <sup>-4</sup>	N/A
Other information	S (DNA) = 2.2 ± 0.1 S (1:1) = 7.3 ± 0.2	ΔH = 10.9 kcal/mol n = 0.95	ΔH = 24.4 ± 2.7 kcal/mol n = 0.34 ± 0.02	Mw (DNA) = 26.1 kDa Mw (Ku) = 144.4 kDa Mw (1:1) = 171.0 kDa Mw (2:1) = 306.2 kDa	N/A	N/A	N/A	N/A	D <sub>h</sub> = 8.9nm	N/A	Rh = 7.1 ± 0.2 nm

905 The last two columns came from additional data: Preliminary SPR obtained by Charbonnier's team, and MDF in  
 906 collaboration with Fluidic Analytics. DNA modified \*FAM or \*\*Biotin.



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