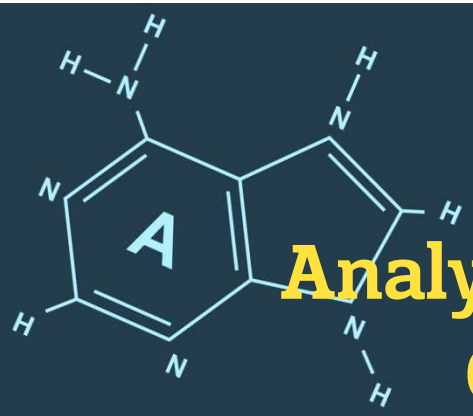
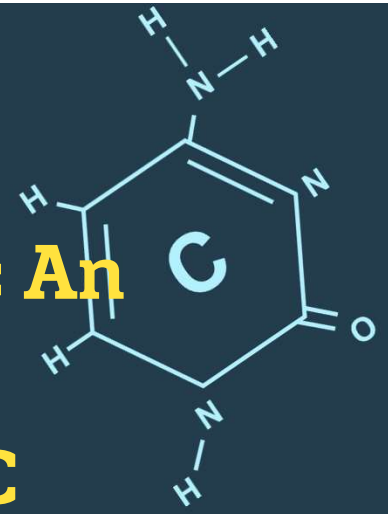




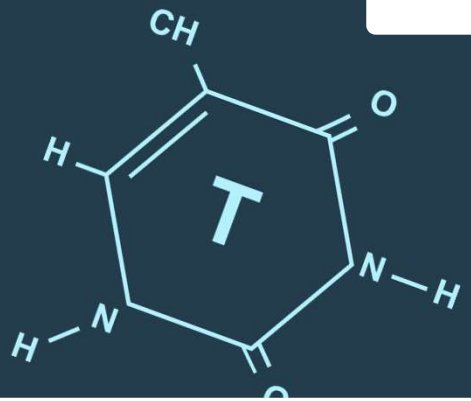
Analytical Ultracentrifugation (AUC): An Overview of the Application of Fluorescence and Absorbance AUC to the Study of Biological Macromolecules

Garrett B. Edwards, Uma M. Muthurajan, Samuel Bowerman, and Karolin Luger

Presented by: Uvindu Abeysekara

BCHM 4000

March 13th, 2024



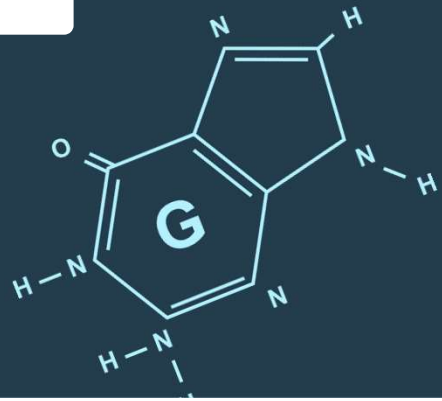


TABLE OF CONTENT:

INTRO TO AUC

- Definition: A biophysical technique to study biological macromolecules in solution
- Purpose: Provides insights into size, shape, and interactions of molecules
- Importance: Widely used in biochemistry and molecular biology research.
- Price of machines are $300,000-$500,000

BASICS OF AUC

- **Principle: Measures sedimentation rates of molecules in a centrifugal field.**
- **Components: Rotor, sample cells, temperature, optical detection systems.**
- **Advantages: Direct measurement in solution state, wide size range coverage. Does not destroy the sample**

Lamm equation:

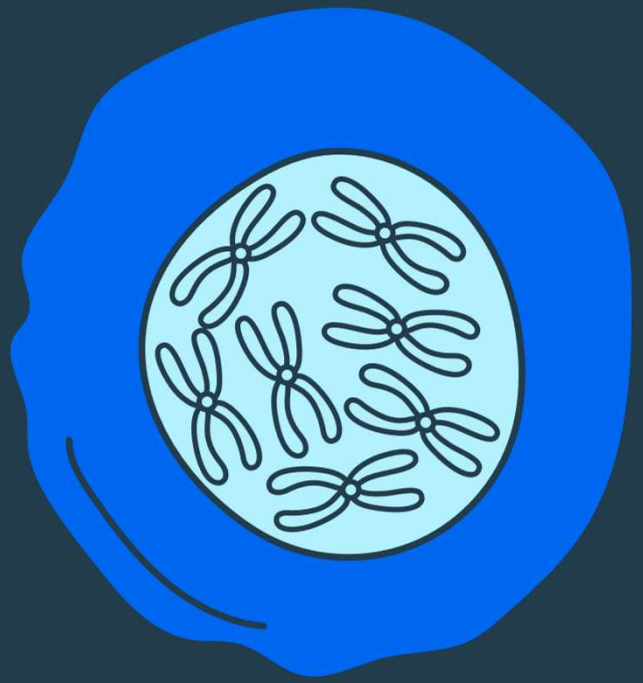
$$\frac{\partial c}{\partial t} = D\left[\frac{\partial^2 c}{\partial r^2} + \frac{1}{r}\frac{\partial c}{\partial r}\right] - s\omega^2\left[r\frac{\partial c}{\partial r} + 2c\right]$$

D = Diffusion coefficient
c = particle concentration
R = radius of cell
t = time
s = sedimentation coefficient

Svedberg equation:

$$\frac{M(1-\bar{v}\rho)}{N_A f} = \frac{v}{\omega^2 r} \equiv s$$

S = sedimentation coefficient
V = solute velocity
R = radial distance from the axis of rotation
ω = angular (rotor) velocity
M = molecular weight



TYPES OF AUC EXPERIMENTS

- **Sedimentation Velocity (SV): Analyzes particle size and shape.**
- **Sedimentation Equilibrium (SE): Determines molecular weight and stoichiometry.**
- **Fluorescence Detection: Enhances sensitivity for low-concentration samples.**

APPLICATIONS OF AUC

- **Protein-Protein Interactions: Studying complex formation and stoichiometry.**
- **Nucleic Acid Analysis: Characterizing DNA-protein complexes.**
- **Drug Development: Assessing binding affinities and kinetics.**

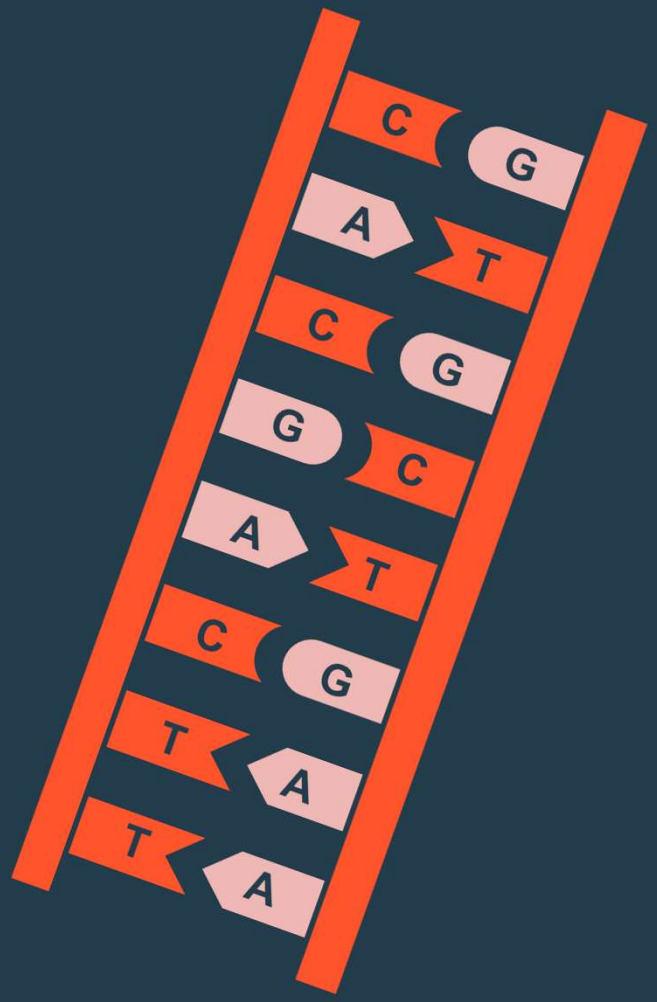


Table 3 2DSA-IT Results for Spn1 in Buffers With or Without 0.1% CHAPS Detergent

	Spn1	Spn1 (0.1% CHAPS)
$S_{20,w}$	2.57 (0.34)[a]	2.56 (0.36)
f/f_0	1.99 (0.56)	2.00 (1.14)
M (Da)	53,107 (31,332)	52,501 (31,574)

[a]Standard deviations are in parentheses. Peaks were integrated to include all S values observed by vHW.

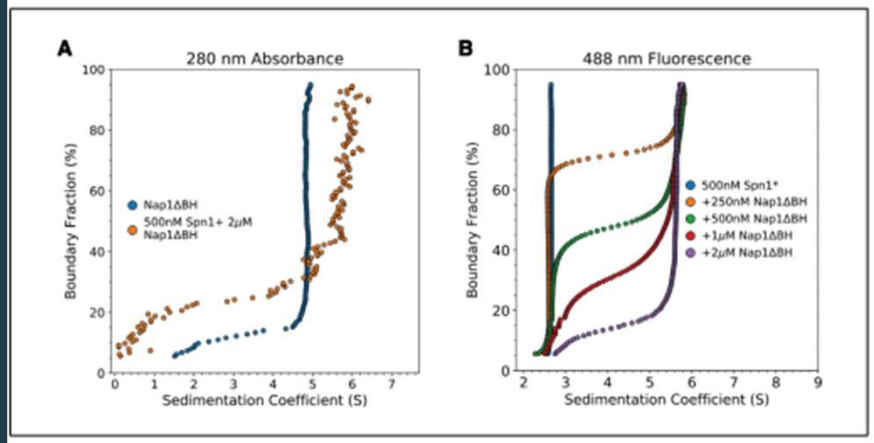


Figure 3 Fluorescence detection simplifies the sedimentation profile of a heterogeneous interacting system. (**A**) G(s) distributions from a 280-nm absorbance SV-AUC experiment. The majority of a sample of Nap1Δ βH (blue) at 10 μM sediments homogenously, whereas addition of 2 μM Nap1Δ βH to 500 nM Spn1 (orange) results in a heterogeneous distribution of at least three states. (**B**) G(s) distributions from an SV-AUC-FDS experiment with Alexa488-labeled Spn1 and varying Nap1Δ βH concentration. Binding results in a shift in fluorescence signal from ~2.6 S to ~5.6 S. The bound complex is more clearly resolved by the FDS than the equivalent sample monitored by absorbance.

Table 4 Results from 2DSA-IT Analysis of Spn1-Nap1Δ βH Samples from Case Study 1

	Spn1 (49.13 kDa)	+250 nM Nap1Δ βH	+500 nM Nap1Δ βH	+1 μM Nap1Δ βH	+2 μM Nap1Δ βH
$S_{20,w}$	2.58 (0.0108)[a]	Solute 1 (70%) = 2.62 (0.55) Solute 2 (18.5%) = 5.89 (n/a)	Solute 1 (43.8%) = 2.63 (0.542) Solute 2 (39.5%) = 5.59 (0.364)	Solute 1 (32.3%) = 2.605 (0.418) Solute 2 (60.6%) = 5.47 (0.434)	Solute 1 (12.9%) = 2.42 (0.586) Solute 2 (78.4%) = 5.63 (0.093)
f/f_0	2.01 (0.0523)	Solute 1 (70%) = 2.14 (0.71) Solute 2 (18.5%) = 2.58 (n/a)	Solute 1 (43.8%) = 2.38 (1.21) Solute 2 (39.5%) = 2.07 (0.5)	Solute 1 (32.3%) = 2.1 (1.9) Solute 2 (60.6%) = 2.18 (0.46)	Solute 1 (12.9%) = 2.17 (0.975) Solute 2 (78.4%) = 1.77 (0.463)
M (Da)	52,640 (2,216)	Solute 1 (70%) = 61,460 (28,731) Solute 2 (18.5%) = 262,188 (n/a)	Solute 1 (43.8%) = 67,772 (31,064) Solute 2 (39.5%) = 180,270 (76,092)	Solute 1 (32.3%) = 58,973 (67,599) Solute 2 (60.6%) = 188,070 (73,371)	Solute 1 (12.9%) = 61,076 (47,392) Solute 2 (78.4%) = 141,480 (51,794)

[a]Measured solute percentages and fitted-parameter standard deviations are in parentheses. Peaks were integrated to include all S values observed by vHW.

CASE STUDY 1: DETERMINING SIZE, SHAPE, AND STOICHIOMETRY

- Example: Analyzing histone chaperones Spn1 and Nap1 interaction.
- Method: Using absorbance and fluorescence AUC for size and stoichiometry determination.
- Results: Understanding the composition and binding sites of the protein complex. Gives much cleaner data
- Theoretical MW: 148.18 kDa
- Experimental MW 141.48 kDa

CASE STUDY 2: MEASURING BINDING AFFINITIES

- Example: quantify the binding affinity between two biological macromolecules, Spn1 and Nap1βH, which was challenging to measure using other methods due to low signal changes upon binding.
- Method: Utilizing FDS to analyze high-affinity binding at nanomolar to micromolar
- Results: The experiment yielded a K_D of approximately 92.1 nM, indicating a strong binding affinity between Spn1 and Nap1βH.

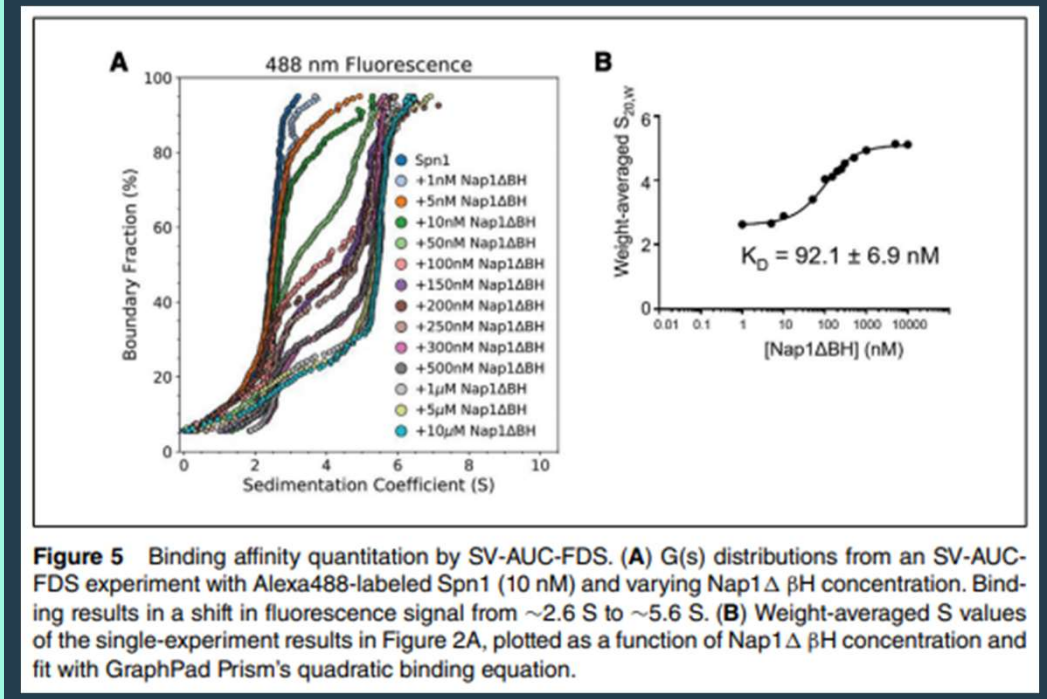


Figure 5 Binding affinity quantitation by SV-AUC-FDS. (**A**) G(s) distributions from an SV-AUC-FDS experiment with Alexa488-labeled Spn1 (10 nM) and varying Nap1Δ βH concentration. Binding results in a shift in fluorescence signal from ~2.6 S to ~5.6 S. (**B**) Weight-averaged S values of the single-experiment results in Figure 2A, plotted as a function of Nap1Δ βH concentration and fit with GraphPad Prism's quadratic binding equation.

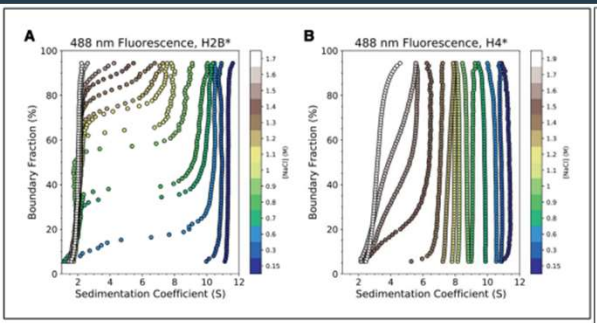


Figure 6 Salt-induced unfolding of eukaryotic nucleosomes monitored by SV-AUC-FDS. (**A**) G(s) distributions from an SV-AUC-FDS experiment with Alexa488-labeled H2B (T112C) included in 147-bp *X. laevis* nucleosomes starting at ~0 M NaCl. Dissociation of H2A-H2B* is observed as a function of ionic strength at ≥0.6 M NaCl. (**B**) G(s) distributions from an SV-AUC-FDS experiment with Alexa488-labeled H4 (E63C) included in 147-bp *X. laevis* nucleosomes starting at ~0 M NaCl. An increasing NaCl concentration results in a decrease in sedimentation between 0.15 and 1.3 M, with dissociation of H3-H4 from DNA at higher salt concentrations.

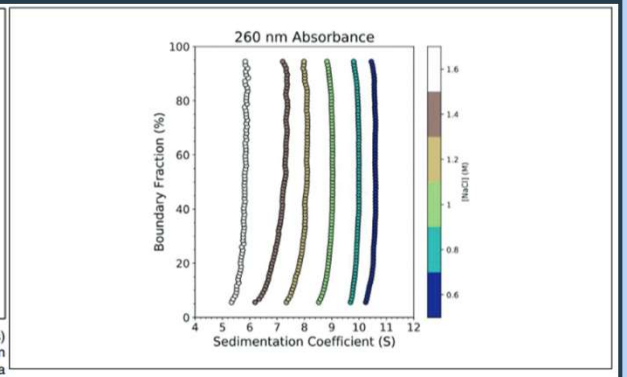


Figure 9 Salt-induced unfolding of eukaryotic nucleosomes monitored by 260-nm absorbance AUC. The G(s) distributions are from an SV-AUC 260-nm absorbance experiment with unlabeled 147-bp *X. laevis* nucleosomes. Nucleosomes were diluted from ~0 M NaCl into the indicated ionic strengths, resulting in nearly homogenous shifts of the DNA-dominated sedimentation signal.

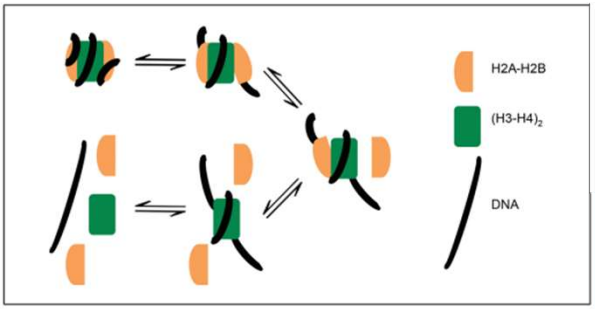


Figure 7 Schematic of the ionic strength–induced nucleosome folding/unfolding process observed in Case Study 3. Folded nucleosomes (top left) first partially unwrap, followed by sequential loss of H2A-H2B dimers. After H2A-H2B dissociation, increasing ionic strength dissociates $(H3\text{-}H4)_2$ from DNA, resulting in a mixture of DNA, H2A-H2B, and $(H3\text{-}H4)_2$. These events are fully reversible, as shown in Figure 8.

Table 6 Frictional Ratios of Partially Unfolded Nucleosome Samples from Case Study 3

	0.15 M NaCl	0.3 M NaCl	0.6 M NaCl	0.7 M NaCl	0.8 M NaCl	0.9 M NaCl	1 M NaCl
f/f_0	1.47 (0.004)[a]	1.56 (0.005)	1.64 (0.006)	1.67 (0.008)	1.74 (0.005)	1.79 (0.061)	1.84 (0.06)

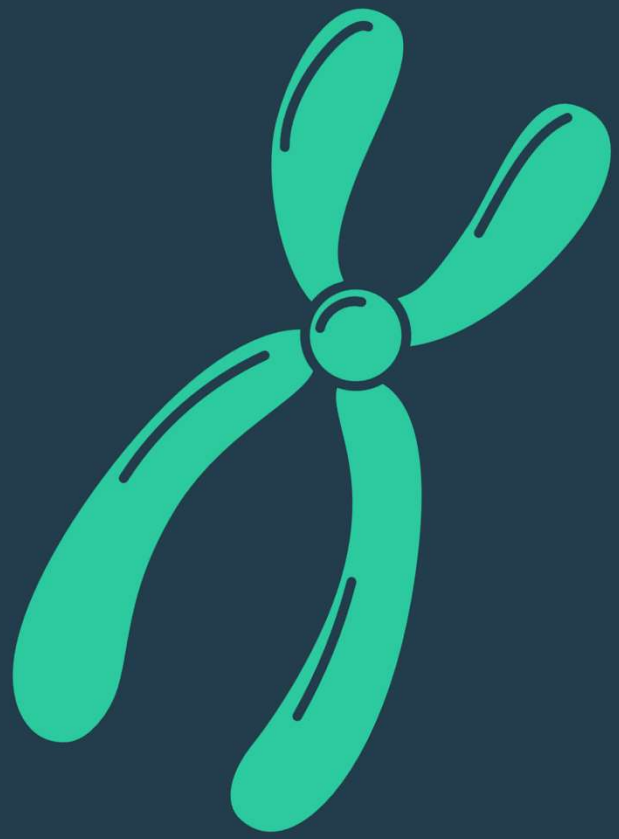
[a]Standard deviations are in parentheses. Values were extracted from 2DSA-IT analysis followed by genetic algorithm–Monte Carlo optimization. Peaks were integrated to include all S values observed by vHW. The observed trend is consistent across three replicates.

CASE STUDY 3: INVESTIGATION OF MACROMOLECULAR ASSEMBLIES

- **Example: Characterization of protein complexes using AUC.**
- **Method: Applying AUC to determine assembly of complexes at various NaCl conc.**
- **Results: Understanding of complex structures and interactions. Number of complexes rough shapes and sizes by also utilizing various frictional ratios.**

DATA ANALYSIS IN AUC

- Importance of User Curation: Ensuring accurate interpretation of experimental data.
- Model Fitting: Evaluating model quality through visual representations and residuals.
- Software Tools: Utilizing UltraScan and SEDFIT for data analysis properly.
- Warning: User bias with limited understanding can falsely represent samples.



FUTURE DIRECTIONS IN AUC RESEARCH

- **Advancements: Continued development of software and instrumentation.**
- **Opportunities: Exploring AUC in diverse biological systems and complex interactions.**
- **Recommendations: Encouraging researchers to consider AUC in their projects, though with proper knowledge of how to apply it.**

CONCLUSION

- Summary: AUC is a valuable tool for studying macromolecular interactions.
- Impact: Enhances understanding of biological systems at the molecular level.
- Limitation: In particular AUC-FDS, requirement of fluorescent tags where 488nm is needed for excitation

REFERENCE

Edwards, G. B., Muthurajan, U. M., Bowerman, S., & Luger, K. (2020). Analytical Ultracentrifugation (AUC): An Overview of the Application of Fluorescence and Absorbance AUC to the Study of Biological Macromolecules. Current Protocols in Molecular Biology, 133(1), e131. https://doi.org/10.1002/cpmb.131

Thank you any question!