class, such as related DNA sequences). In interaction processes that are complicated, there can be multiple binding sites, cooperative interactions, and so forth [1-3]. In order to determine the equilibrium and/or kinetic constants for binding, all techniques must factor the concentrations of P and T into the concentrations of free P and T, on the left side of the equation, as well as the concentrations in C, on the right side of the equation. This evaluation can be achieved by various methods, including equilibrium dialysis, spectral measurements, gel shift, calorimetry, DNase I footprinting, and related techniques [1-4]. Many of these methods require labeling of P or T with a fluorescent or radioactive tag.

A recent development in instruments that investigate biomolecular interactions in label-free mode, is surface plasmon resonance (SPR) detection with a biospecific sensor chip [1-4]. In BIACORE technology, the sensor chip is created by applying a thin layer of gold (~50 nm) to a glass surface (Figure 1, left). In the most common type of sensor chip, carboxymethyl-dextran is linked to the gold to give the interaction layer (~100 nm thick). One of the interacting molecules, either T or P, must be linked to this layer to create the biospecific recognition surface. Because SPR responds to changes in refractive index (Figure 1, right) and, thus, to changes in mass, it is advantageous to attach the molecule with the lowest molecular weight to the surface.

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Monday, June 26, Session II

L5

THERMOFLUOR ASSAY: HOW TO MAKE YOUR PROTEIN HAPPY

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Thermofluor assay, also known as differential scanning fluorimetry or thermal shift assay, is a fluorescence-based biophysical method used to assess protein thermostability. Temperature gradient is applied on protein sample in the presence of a hydrophobic fluorescent dye, which binds to the hydrophobic core of the protein exposed during the unfolding process, and fluorescence signal is recorded. Melting temperature, determined from the protein melting curve, is a measure of protein stability in the given environment.

Thermofluor was originally developed for high-throughput screening for ligands [1], but the application of this versatile technique is not limited to drug discovery. It can be used for monitoring of protein-protein interactions [2], assessment of protein-ligand affinity [3], identification of protein function [4], evaluation of properties of protein constructs in engineering [5], or optimization of purification procedure [6].

Thermofluor assay is a key technique of structural biology [7]. Identification of buffer conditions or additives stabilizing the protein prior to crystallization greatly improves the hit rate in initial crystallization screening trials. Thermofluor-based buffer optimization can also give rise to alternative crystal forms with improved diffraction quality. Application of thermofluor will be demonstrated on example cases.

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L6

BIOMOLECULAR INTERACTIONS ANALYTICS USING MICROSCALE THERMOPHORESIS

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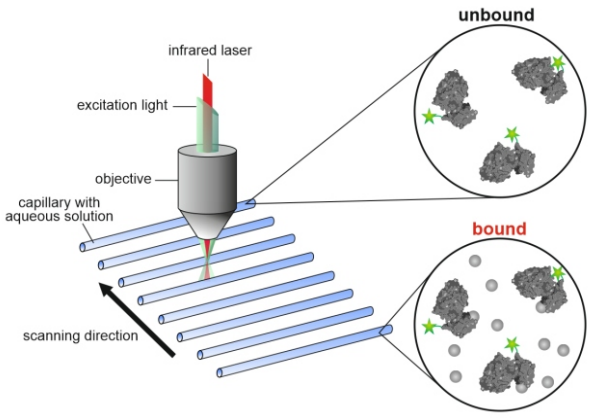
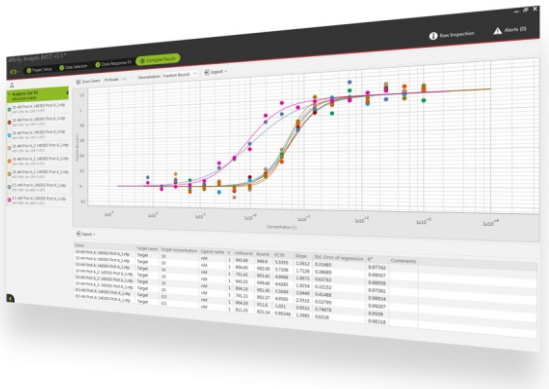
NanoTemper Technologies proprietary technology; Microscale Thermophoresis (MST) allows for quantitative analysis of biomolecular interactions in free solutions. The technique is based on phenomenon of thermophoresis, the directed motion of molecules in temperature gradient. Thermophoresis is highly sensitive to all types of binding-induced changes of molecular properties, be it in size, surface charge and hydration shell or conformation. Upon any type of interaction at least one of the above mentioned parameters will change. In an all optical approach, MST is induced using an infrared laser for local thermal gradient induction, and molecules mobility across it is analysed through fluorescence detection. In addition to fluorescence by various available fluorophores or fusion proteins attached to one of the binding partners, intrinsic protein fluorescence can be utilized for MST thus allowing for label-free MST analysis.

Our technique's flexibility in assay design and experimental execution qualifies MST for biomolecular interaction analysis in complex experimental settings, which we herein demonstrate by addressing typically challenging types of binding events from various fields of life science. Furthermore, MST assays are highly adaptable to fit to the diverse requirements of different biomolecules, e.g. membrane proteins stabilized in solution, multi-component protein structures or nanoparticles. The type of buffer and additives can be chosen freely and does not discriminate its composition. Measuring is also possible in complex bioliquids like cell lysate, blood serum or even direct analysis without protein purification and thus under close to in vivo conditions. Binding modes that are quantifiable via MST include dimerization, cooperativity and competition as well. Thermophoresis makes both high- (pM) and low affinity (mM), interactions available in shape of single instrument solution. For more insight and details you are welcome to visit our booth.





L7

SEDIMENTATION ANALYSIS OF PROTEINS USING ANALYTICAL ULTRACENTRIFUGE: OLD METHOD WITH NEW HORIZONS

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Sedimentation analysis of macromolecules carried out in analytical ultracentrifuge is a powerful method for the study of proteins, nucleic acids and other polymers and their various complexes. Monitoring sedimentation of macromolecules in the centrifugal field allows their hydrodynamic and thermodynamic characterization in solution, i.e. in native conditions, without interactions with any matrix or surface. This allows direct measurement of molecular weight and sedimentation coefficient of macro-mo lecules, monitoring of sample purity and homogeneity, prediction of size and shape of sedimenting species and, last but not least, study of equilibrium reactions, including determination of their stoichiometry and equilibrium constants.

In the present lecture, we will first focus on the history physical principles of the technique and also on the properties and potential of a modern instrumentation. Two types of experiments performed using analytical ultracentrifuge (i.e. sedimentation velocity and sedimentation equilibrium) will be discussed, together with a brief introduction into sedimentation theory. In the end, sedimentation data analysis will be described and examples of utilization of analytical ultracentrifugation in biomolecule research will be provided.

Combination of new instrumentation and computational software for data analysis has led to major advances in characterization of proteins and their complexes. After temporary silence in the past decades, analytical ultracentrifugation at presence experiences renaissance in proteomic and structural biology research while still being heavily used e.g. for characterization of aggregation of monoclonal antibodies in biopharmaceutical industry.

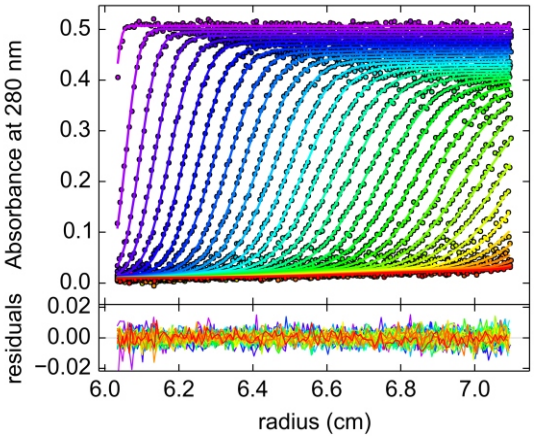

Figure 1. Sedimentation velocity (SV) analysis of recombinant human proliferating cell nuclear antigen (PCNA); fitted data (top) and residuals plot (bottom).

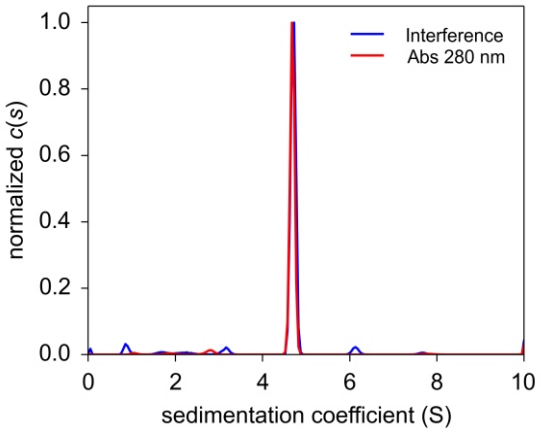

Figure 2. Distribution of sedimenting species for the PCNA SV analysis performed with both absorbance and interference optics showed it is a trimer

L8

CIRCULAR DICHROISM SPECTROSCOPY OF PROTEINS

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Structure plays a key role in protein's function. Characterization of newly isolated or genetically modified proteins therefore often begins by verification of their proper folding. Spectroscopic techniques represent the first-choice methods used to analyse the conformational behaviour of biomolecules under different conditions. They are also used for comparison of the structural properties of related molecules such as homologous or mutant forms of proteins. Furthermore, spectroscopic methods are used to determine the structural stability of proteins and to monitor the kinetics of their structural transitions under different physicochemical conditions (temperature, pH, chemical denaturants or organic co-solvents). An overview of spectroscopic techniques used for analysis of secondary and tertiary structure of proteins will be presented in the lecture. Main attention will be paid to the circular dichroism (CD) spectroscopy [1-3].

CD spectroscopy is a fast, quantitative and non-destructive spectroscopic technique where the CD of molecules is measured over a range of wavelengths. CD spectroscopy is used to study chiral molecules of all types and sizes. A primary use is in analysing the secondary structure or conformation of macromolecules, particularly proteins. As protein secondary structure is sensitive to its environment, CD can be used to observe how secondary structure changes with environmental conditions or interaction with other molecules. Structural, kinetic and thermodynamic information about macromolecules can be derived from CD spectroscopy. The principle of CD spectroscopy, attainable types of structural information, experimental aspects, advantages/disadvantages and specific examples of applications will be introduced and discussed in this lecture.

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PL1

ROLE OF THE N-TERMINAL REGION IN RYANODINE RECEPTOR CHANNEL ACTIVATION

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Mutations in the cardiac ryanodine receptor (RyR2), the ion channel responsible for release of calcium ions from intracellular stores into cytoplasm, are the cause of several inherited cardiac arrhythmias. At themolecular level, disease symptoms can be mimicked by domain peptides from mutation-prone regions of RyR2 that bind to RyR2 and activate it. Here we show that the domain peptide DPcpvtN2, corresponding to the central helix of the N-terminal region of RyR2, activates the RyR2 channel. Structural modelling of interaction between DPcpvtN2 and the N-terminal region of RyR2 in the closed and open conformation provided three plausible structures of the complex. Only one of them could explain the dependence of RyR2 activity on concentration of DPcpvtN2. The structure of the complex was at odds with the previously proposed "domain switch" mechanismof competition between domain peptides and ryanodine receptor domains. Likewise, in structural models of the N-terminal region, the conformational changes induced by DPcpvtN2 binding were different from those induced by mutation of central helix amino acids. The activating effect of DPcpvtN2 binding and of mutations in the central helix could be explained by their similar effect on the transition energy between the closed and open conformation of RyR2.