Automated, Low Volume Dynamic Light Scattering Technology to Accelerate Protein Crystallization

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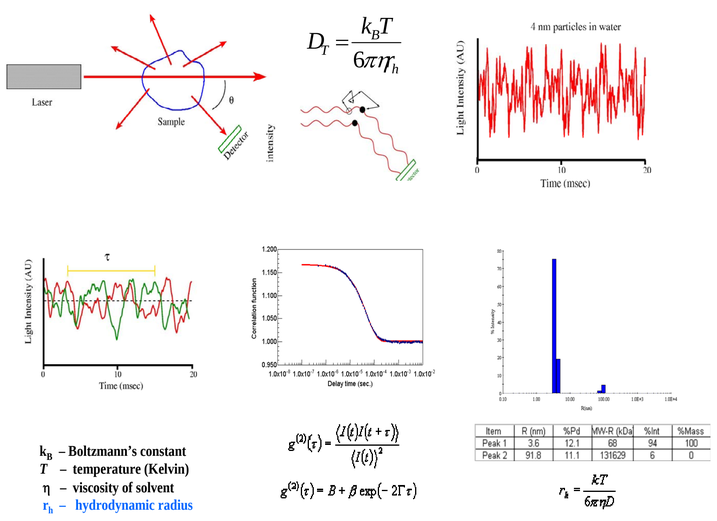
Introduction

- The quest for solving protein structure largely relies upon X-Ray diffraction, a method requiring crystalline forms of the target protein. Among the many steps comprising structure determination, the process of protein crystallization represents one of the most significant, time-consuming challenges. A new low sample volume, automated dynamic light scattering (DLS) technology has been developed – the DynaPro Plate Reader. The DynaPro Plate Reader can measure protein samples less than 5 microliters in volume, at less than 1 mg/mL concentrations in 1536 well-plate formats. By analyzing 100's of samples per day, in an unattended fashion, the DynaPro Plate Reader improves the productivity of vital dynamic light scattering applications in the protein crystallization process: screening proteins for aggregation prior to crystallization trials and solubility screening. More conditions can be explored in less time with less sample, leading to the successful optimization of the protein and/or crystallization conditions.

A brief review of dynamic light scattering



k_B – Boltzmann's constant
T – temperature (Kelvin)
η – viscosity of solvent
r_h – hydrodynamic radius

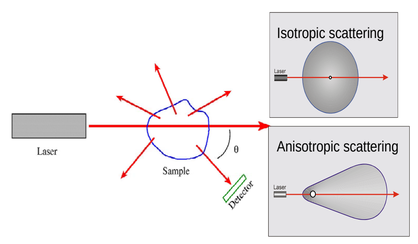
$$g^{(2)}(\tau) = \frac{\langle I(t)I(t+\tau)\rangle}{\langle I(t)\rangle^2}$$

$$g^{(2)}(\tau) = B + \beta \exp(-2\Gamma\tau)$$

Item	R (nm)	%Pd	MW-R (kDa)	%Int	%Mass
Peak 1	3.6	12.1	68	94	100
Peak 2	91.8	11.1	131629	6	0

$$r_h = \frac{kT}{6\pi\eta D}$$

And Multi-angle static light scattering



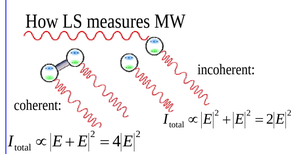
small particles Rg ~ < λ/20

$$I_{scattered} \propto Mc\left(\frac{dn}{dc}\right)^2$$

large particles Rg > λ/20

$$I(\theta)_{scattered} \propto Mc\left(\frac{dn}{dc}\right)^2 P(\theta)$$

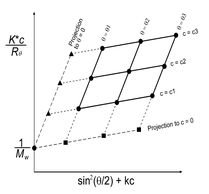
How LS measures MW



$I_{total} \propto |E+E|^2 = 4|E|^2$

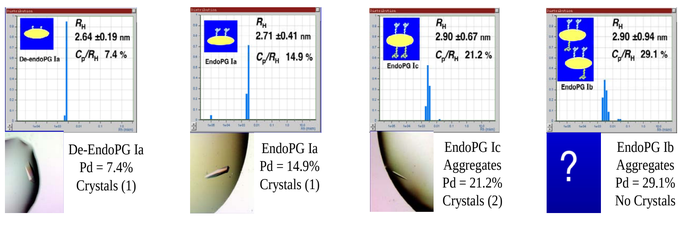
$I_{total} \propto |E|^2 + |E|^2 = 2|E|^2$

$$\frac{K^* c}{R(\theta,c)} = \frac{1}{M_w P(\theta)} + 2A_2 c$$



- If proteins are 'isotropic', why multi-angle light scattering?
- Higher Precision, Built-in Redundancy, Greater Versatility

DLS as a Predictor of Crystallization



De-EndoPG Ia Pd = 7.4% Crystals (1)

EndoPG Ia Pd = 14.9% Crystals (1)

EndoPG Ic Aggregates Pd = 21.2% Crystals (2)

EndoPG Ib Aggregates Pd = 29.1% No Crystals

DynaPro DLS size distributions and measured Polydispersity (Pd) from the pre-crystal screening of four forms of Endopolygalacturonase (EndoPG I). The samples with lower polydispersity were more readily crystallized compared to samples with higher polydispersity. Original DynaPro data, © Hiroaki KATO, Ph.D., Professor, Structural Biology, The Graduate School of Pharmaceutical Sciences, Kyoto University, 46-29, Yoshida-Shimoadachi, Sakyo, Kyoto 606-8501, JAPAN. Used with permission. Crystallization conditions: (1) 15% PEG 4000, 0.2 M NaCl, 50 mM Acetate buffer, pH 5.0 (2) 18% PEG 8000, 0.2 M Ca Acetate, 0.1M Cacodylate Buffer, pH 6.5.

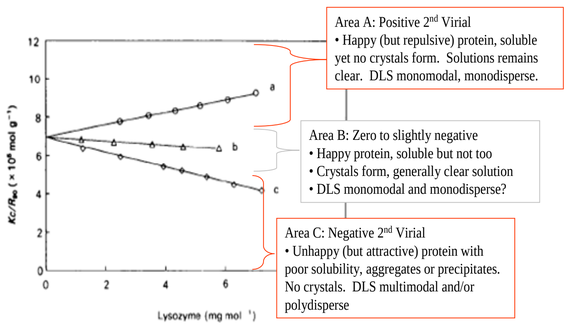
Size Distribution	Crystal Grown	No Crystal	Total	% Success	% Failure	S/F Ratio
Narrow unimodal	34	10	44	77%	23%	3.40
Broad unimodal	6	4	10	60%	40%	1.50
Multi-modal	1	11	12	8%	92%	0.09
Total	41	25	66			

- A. D'Arcy, "Crystallizing proteins - a rational approach?", Acta Crystallographica D50, 469-471 (1994).

➢ The "D'Arcy" assay tests protein in dilute, simple buffer conditions (e.g. PBS) – not in crystallization conditions with precipitating agents. If protein is not monomodal, clone and express modified protein until it is monomodal. Then proceed with crystallization trials.

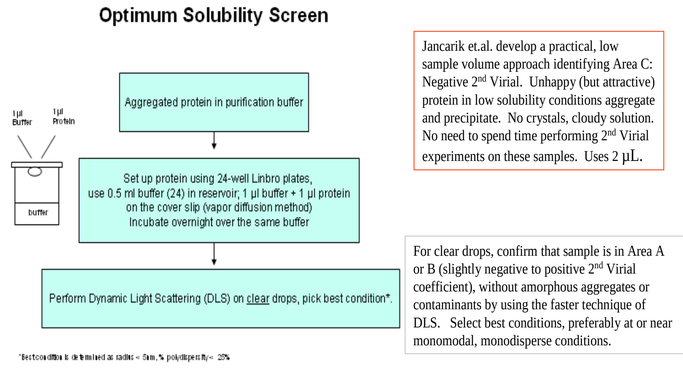
2nd Virial as a Predictor of Crystallization

"A. George, W. W. Wilson", "Predicting protein crystallization from a dilute solution property", *Acta Crystallographica* **D50**, 361-365 (1994).



2nd Virial data from Wyatt Technology's 18-angle MALS "DAWN" system with flow cell (to avoid error introduced by optical misalignments associated with batch configurations). Why aren't 2nd Virial coefficients more widely used? Takes a long time and requires larger volumes of sample. Sample volumes are 100's of µL per condition, with each condition requiring characterization of 6-10 dilutions, under crystallization conditions, for a typical duration of 1 to 2 hours per condition.

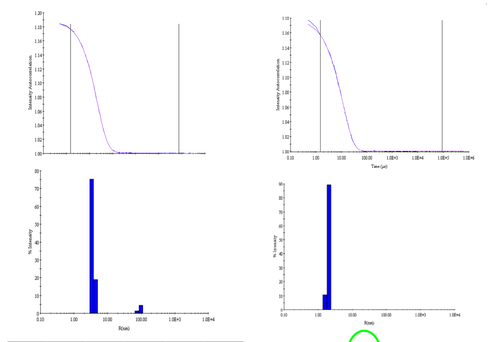
An Optimal Solubility Screen: A visual solubility test combined with dynamic light scattering



- "J. Jancarik, R. Pufan, C. Hong, S.-H. Kim, R. Kim", "Optimum solubility (OS) screening: an efficient method to optimize buffer conditions for homogeneity and crystallization of proteins", *Acta Crystallographica* **D60**, 1670-1673 (2004).

DLS Plate Reader Results from known, crystallizable protein.

- Protein SEC (Soluble, Easy to Crystallize protein) was screened with the Plate Reader in 30 crystallization buffers in less than one hour. A one µL drop of the 14.3 kD protein stock solution (13.5 mg/mL in 10mM Hepes, pH 7.5, 100 mM NaCl) was diluted with a four µL drop of the screening solution, and allowed to equilibrate in the plate for one hour. Shown below, right hand side, is the best (judged by DLS data) of several conditions that produces a monomodal, monodisperse size distribution. Ten, one second acquisitions were averaged together to form the autocorrelation function, which is fitted with a proprietary regularization algorithm. The relative polydispersity, %Pd is below 15%, indicating a homogeneous protein population. BSA, 2 mg/mL, 4 µL volume is shown for comparison purposes (left hand side).



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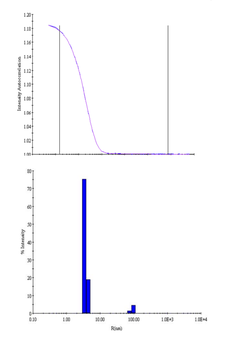
Item	R (nm)	%Pd	MW-R (kDa)	%Int	%Mass
Peak 1	2	7.8	17	100	100

DLS Plate Reader results from known, uncrystallizable protein.

- Protein ISNOC (insoluble, never crystallized), 49 kD, was screened in over 800 conditions in 3 days using less than 400 µL of protein stock solution. 0.5 µL drops of stock solutions taken from clear crystallization drops and diluted with 4 µL of mother liquid and screened in the DynaPro Plate Reader. A typical size distribution from this protein, which has yet to be crystallized, is shown on the right hand side – the classic multi-modal size distribution associated with protein aggregation. A BSA sample, 2 mg/mL, 4 µL volume is shown for comparison purposes (left hand side). The exhaustive search led to a set of most promising conditions, judging by DLS, which upon modification have resulted in small crystals.



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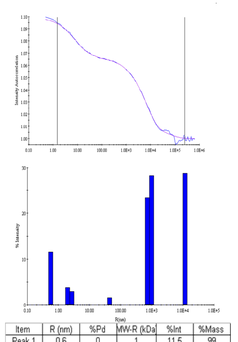
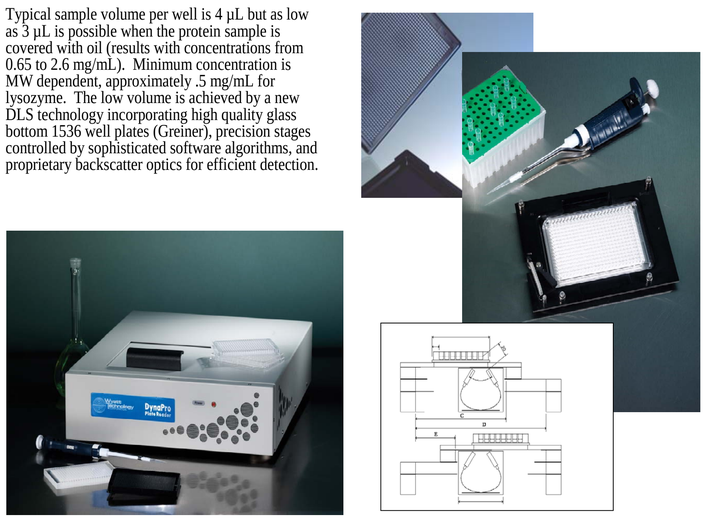


Item	R (nm)	%Pd	MW-R (kDa)	%Int	%Mass
Peak 1	0.6	0	1	11.5	99
Peak 2	2.3	15.6	24	6.7	1
Peak 3	44.6	0	24293	1.5	0
Peak 4	876.5	15.1	2585700	51.6	0
Peak 5	11995	0	1.176E+10	28.7	0

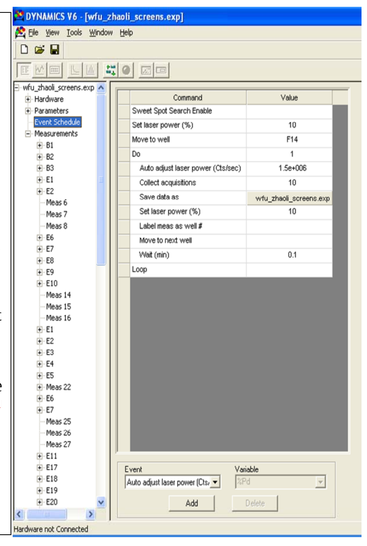
Low Sample Volume DLS Plate Reader Technology

- Typical sample volume per well is 4 µL but as low as 3 µL is possible when the protein sample is covered with oil (results with concentrations from 0.65 to 2.6 mg/mL). Minimum concentration is MW dependent, approximately .5 mg/mL for lysozyme. The low volume is achieved by a new DLS technology incorporating high quality glass bottom 1536 well plates (Greiner), precision stages controlled by sophisticated software algorithms, and proprietary backscatter optics for efficient detection.



Fully Automated, Software Controlled

- Enjoy all the benefits of DLS …
  - a technique that is fast and easy to use:
  - The sample is not perturbed – the novel technology measures the sample directly through the plate without any liquid handling;
  - The sample is fully recoverable – using standard pipettors or liquid handlers;
- *Coupled with automation*
  - The Plate Reader is fully automated by the Dynamics software, with proprietary built-in optimization algorithms, able to measure hundreds of conditions in a single day…unattended.
  - The ability to walk away from the instrument will save hours of time standing by and waiting for conventional batch DLS instrument.
  - Remove samples and discard the inexpensive disposable plates – No time consuming clean up required!
  - Spend more time searching more conditions, and analyzing valuable data.
- ***Orders of magnitude more efficient than conventional DLS batch systems…***



Sample Preparation

- Sample Volume, Concentration, Consumption and Loading
  - Minimum measured concentration for 14kD protein lysozyme is approximately .5 mg/ml provided the sample volume is 7 µL. At typical concentrations of DLS assays (1-2 mg/mL or higher), the sample volume required is 3 µL (sample under oil) or 4 µL (typical). Samples prepared with 3.5 µL mother liquid + 0.5 µL protein stock solution or 4.0 µL mother + 1.0 µL protein.
  - For a full plate of 1536 wells x 4 µL per well = 6144 µL sample volume is required, which requires a total of ~ 12 mg of protein assuming 2 mg/mL protein concentration. However, a full plate is not required. We recommend preparing and measuring a 'manageable' number of samples on the order of 48, 96, 192 at a time. The time to measure each well including acquisition and optimization is approximately 1 minute, requiring about 20 hours for a full 1536 plate; 6.5 hours for 384 samples; 1.5 hours for 96 samples.
  - Samples were loaded manually using P10 or P20 with gel loading tips. Sample can be loaded into the plate using appropriate liquid handling robots.
- Sample Evaporation
  - When measuring large numbers of samples (e.g. 1536 plates) or when measuring with 'large diameter' plates e.g. 96 and 384 formats, evaporation does become an issue for wells near the end of the measurement cycle. In the 1536 /low volume plate format experiments demonstrate that covering the sample with a drop of silicon oil avoid evaporation and do not interfere with the DLS measurements (adding oil enables 3 µL sample volumes). Alternatively, after loading samples, covering the samples with clear tape (Hampton Research) reduces evaporation. The tape must be removed prior to measurements with the "Lo-base" Greiner MicroPlates as the tape interferes with the measurement. We recommend uncovering one to four rows at a time and keeping any remaining wells covered until it is time to measure. Standard plate lids provided with the microplates are not much use in preventing evaporation.

Summary

- The DynaPro Dynamic Light Scattering Plate Reader is a novel combination of low sample volume capability with an automated batch approach useful for screening protein and/or solution conditions prior to protein crystallization.
- Hundreds of samples can be measured in less than one day, without user involvement, affording a significant time savings compared to conventional one-at-a-time batch DLS systems.
- Sample volumes as low as 3 µL can be performed under oil, providing the capability to measure more conditions or performing redundant experiments for greater confidence in DLS results.

PLATE READER BENEFITS		
Description	**Batch (Cuvette) Operation**	**Plate Reader (Microplate)**
Time to prepare 384 samples (make dilutions, measure salt concentrations, etc.)	2-16 hours	<2 hours
Loading samples	>12 hours (including time to insert, remove, and clean each cuvette)	<1 hour
Collecting data, personnel required	>6 hours, and a person required to change samples	No personnel required
Totals	20-34 hours	<3 hours

The DynaPro Plate Reader dramatically improves your productivity by over a factor of 10 compared to conventional batch DLS systems. Accelerate your research timetable, explore more conditions, or simply make better use of time with the Plate Reader.

Wyatt Technology United Kingdom Ltd.