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## Structural Studies of Macromolecules in Solution using Small Angle X-Ray Scattering

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Dear Indrani

I am writing to submit manuscript No. JoVE57906 "Structural Studies of Macromolecular Interactions in Solution" for your consideration for a special issue of JoVE on "Protein-protein Interaction Methods".

Thank you for your considerations.

Sincerely,

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**TITLE:**

# Structural Studies of Macromolecules in Solution using Small Angle X-Ray Scattering

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**KEYWORDS:**

Extracellular matrix protein, hybrid solution structure, laminin, multidomain protein, nidogen, protein interaction, protein structure, structural domain, small angle X-ray scattering, three-dimensional model

**SUMMARY:**

Here, we present how Small Angle X-Ray Scattering (SAXS) can be utilized to obtain information on low-resolution envelopes representing the macromolecular structures. When used in conjunction with high-resolution structural techniques such as X-Ray Crystallography and Nuclear Magnetic Resonance, SAXS can provide detailed insights into multidomain proteins and macromolecular complexes in-solution.

**ABSTRACT:**

Protein-protein interactions involving proteins with multiple globular domains present technical challenges for determining how such complexes form and how the domains are oriented/positioned. Here, a protocol with the potential for elucidating which specific domains mediate interactions in multicomponent system through *ab initio* modeling is described. A method for calculating solution structures of macromolecules and their assemblies is provided

that involves integrating data from small angle X-ray scattering (SAXS), chromatography, and atomic resolution structures together in a hybrid approach. A specific example is that of the complex of full-length nidogen-1, which assembles extracellular matrix proteins and forms an extended, curved nanostructure. One of its globular domains attaches to laminin $\gamma$-1, which structures the basement membrane. This provides a basis for determining accurate structures of flexible multidomain protein complexes and is enabled by synchrotron sources coupled with automation robotics and size exclusion chromatography systems. This combination allows rapid analysis in which multiple oligomeric states are separated just prior to SAXS data collection. The analysis yields information on the radius of gyration, particle dimension, molecular shape and interdomain pairing. The protocol for generating 3D models of complexes by fitting high-resolution structures of the component proteins is also given.

**INTRODUCTION:**

Cells contain intricate networks of proteins that act as molecular machines to carry out cellular functions such as signaling cascades and maintaining structural integrity. The ways in which these different components move and interact in three-dimensional space gives rise to the specific functions of the macromolecules. The importance of protein structure, dynamics, and interactions in determining function has provided the need for continually evolving, complex techniques to measure these properties. Of these, Nuclear Magnetic Resonance (NMR), X-Ray Crystallography (XRC) and more recently, Cryo-Electron Microscopy (CEM) provide high-resolution structural information. However, XRC and CEM yield structures of one of many biomolecular states and lack information about the dynamics of the protein structure, while 3D structure determination by NMR is typically limited to smaller globular proteins. One way to overcome these limitations is to utilize Small Angle X-Ray Scattering (SAXS) to generate molecular envelopes of large, multidomain, or complexed systems, and combine the high-resolution rigid macromolecular structures to elucidate the global architecture and dynamic features.

SAXS produces low-resolution envelopes of macromolecular complexes with a resolution of approximately 10-20 Å [1], giving insight not only into the structure but also the dynamic characteristics that the complex displays. Although SAXS utilizes X-rays to uncover molecular structure, it is unlike XRC in that the random isotropic orientation of the particles in solution does not lead to diffraction, but rather to scattering, which cannot yield atomic resolution. Instead, an electron "envelope" of the macromolecule is generated that represents an average of the conformations that the macromolecule displays. This information can be used in direct fitting of previously solved atomic resolution structures to infer regions of flexibility in a single protein or subunit organization, or dynamics in a larger, multi-protein complex. SAXS data is collected at synchrotrons using high-energy monochromatic X-rays or from in-house sources, which offer a weaker X-rays source requiring hours rather than seconds of sample exposure time (**Figure 1**). SAXS data is often collected from several samples with a single experimental setup and buffer, requiring an extended time to collect a round of useful data on a system. Samples should, therefore, be stable and non-aggregating for at least a few hours based on verifiable quality control methods such as dynamic light scattering (DLS) and/or analytical ultracentrifuge (AUC) analysis to obtain high-quality SAXS data[2,3]. Here we provide a practical description of SAXS, the

principles behind its usage, benefits, limitations and sample preparation and focus heavily on data collection and analysis, along with touching briefly on *ab initio* modeling using the extracellular matrix proteins nidogen-1 and laminin $\gamma$-1 as an experimental example.

**Principles, Benefits, and Limitations of SAXS:**

The guiding principle(s) behind SAXS is relatively simple: a solution of the monodispersed preparation of macromolecule(s) of interest is placed within a capillary and is exposed to a high energy monochromatic X-ray beam. The photons cause electrons of the atomic shell to begin oscillating, resulting in a spherical wave being emitted of the same energy and wavelength. Since every electron will oscillate, a constant background will be achieved, and the resulting electron density of the macromolecule is contrasted to the background. The resulting scattering intensity is collected as a function of the scattering angle, 2Θ (**Figure 1**).

While other techniques such as XRC, NMR, and CEM provide structural information at the atomic level, there are multiple benefits to SAXS that other techniques cannot provide. SAXS can be performed in almost any buffer and does not require any special sample preparation. This is particularly important in studying the behavior and structure of macromolecules under varying conditions, such as the presence or absence of mono- or divalent cations or changes in pH[4,5]. SAXS has the ability to provide information about flexible regions of a macromolecule[6], something the other listed techniques can struggle with. Therefore, SAXS can be used as a strong complimentary technique with the stable portions of a macromolecule being studied with XRC, NRM or CEM, and the entire macromolecule or complex analyzed in low resolution with SAXS and combined using various analysis tools such as FoXSDock[7] or CRYSOL[8]. Since SAXS is a solution technique, it is often used to confirm if static structures such as those obtained from XRC are consistent in solution[6]. SAXS also has the advantage of being a technique that requires a relatively small amount of sample investment (typically 50-100 µL) and a relatively small amount of experiment time (30 min-1 h).

The largest limitation of SAXS is the vulnerability to sample aggregation and/or degradation, which can lead to incorrect structural predictions. An aggregation, even as low as 5%, can scatter light in very high amounts, leading to an overestimation of the maximal particle dimension ($D_{max}$) and radius of gyration ($R_g$). On the other hand, sample degradation can lead to an underestimate of molecular properties. This vulnerability arises from SAXS being an averaging technique, which means that sample homogeneity is critical to achieving reliable and reproducible results. Any sample that is to be analyzed by SAXS should, therefore, undergo multiple methods of purification and homogeneity checks, such as denaturing and native gel electrophoresis, size exclusion chromatography, dynamic light scattering, and analytical ultracentrifugation. Often, SAXS beamlines will run samples through high-performance liquid chromatography as a final quality control step before SAXS (S-SAXS)[3,9]. SAXS data should be collected at multiple concentrations and the $R_g$ of each data set should be compared, ensuring a close similarity to avoid interparticle interactions and aggregation, which results in an overestimation of particle dimensions, leading to inaccurate data analysis and modeling. Since scattering depends on both concentration and size, smaller macromolecules may require a more specific optimization of the

concentration range. This is due to the *Reciprocity Theorem*, where large sizes scatter towards small angles and small sizes towards large angles. This manifests in data collection, where $I_O$ is proportional to $R^6$, where R is the particle radius. A final limitation of SAXS is the potential for radiation damage to the sample during exposure, which can lead to distortion of the data. It is good practice to compare sample quality before and after SAXS sample exposure to ensure this is not occurring.

**PROTOCOL:**

**1. SAXS Sample Preparation and Data Acquisition**

1.1. Sample Preparation:

1.1.1. SAXS experiments require homogeneous, stable and non-aggregating protein samples; observe stability and oligomeric state with size exclusion chromatography (S), DLS and/or AUC prior to data collection.

1.1.2. Subject samples (nidogen-1 and laminin $\gamma$-1 in this case) to DLS analysis and tricine SDS-PAGE to visualize sample purity[10].

Note: Samples may cover a range of concentrations (1-4 mg/mL) depending on their size, their solution behavior such as self-association and aggregation along with stability; here, five concentrations of nidogen-1, (139 kDa), three of laminin $\gamma$-1 (109 kDa) and four of the S-purified equimolar complex were prepared as previously described[10].

1.2. Data Collection:

1.2.1. Collect SAXS data using an in-house system or synchrotron, according to manufacturer's or facility guidelines.

Note: Data used in this work was collected using an in-house system (see **Table of Materials**) that contains a 3-pinhole camera equipped with + 002 microfocus sealed tube (Cu Kα radiation at 1.54 Å) and Confocal Max-Flux (CMF) optics operating at 40 W. The system is also equipped with a 200 nm multi-wire 2D detector for data collection. However, with the availability of modern synchrotrons in France, Germany, UK, USA, and other countries, which provide access to an S-SAXS set-up that facilitates separation of a monodispersed preparation from possible aggregation/degradation, we now routinely collect data at synchrotron facilities. A recently published article on a DNA G-quadruplex[11] is an example of an S-SAXS data collection strategy. In this case, the SAXS data were collected in the range of $0.08 \leq q \leq 0.26$ Å for 3 hours for nidogen-1 (2.0, 2.5, 3.0, 3.5 and 4.0 mg/mL); the laminin γ-1 (1.5, 2.0 and 2.5 mg/mL) and their complex (0.8, 1.0, 1.25 and 1.5 mg/mL).

1.2.2. Reduce data for buffer and samples using processing software specific to the system. Subtract the buffer contribution from protein data using a program like PRIMUS/qt[12] (**Figure 2A**).

## 2. **Data Analysis**

Note: Currently, there are a few software packages that are useful for SAXS data analysis: ScÅtter[43] (download available at www.bioisis.net), bioXtas RAW[44], and the ATSAS suite[13]. This section provides an overview of general steps to be taken when analyzing raw SAXS data using the ATSAS program suite and specific steps are taken from the ATSAS 2.8.1 download. Other programs can be used and are briefly discussed later.

### 2.1. Buffer Subtraction

Note: These steps are relevant for static SAXS samples only.

2.1.1. Select the "TOOLS" menu option in PRIMUS/qt and select the data files of interest (up to 13 at once) using the "SELECT" button. Be aware that data files must be in ASCII format, in which the first column is the s-vector axis and the second column is the intensity. Repeat this step for data collected for the buffer itself by inserting this data into a second "TOOLS" menu.

2.1.2. Select "SUBTRACT" in the Data Processing window, which will generate a subtracted scattering curve representing only scattering from the macromolecule of interest. Repeat this step for each concentration.

### 2.2. Guinier Analysis

2.2.1. To perform Guinier analysis, load a buffer subtracted scatter curve into PRIMUS/qt as previously described in step 2.1.1.

2.2.2. Click "GUINIER," which will proceed in opening the Primus Guinier Wizard; a plot of $ln(I)$ vs $q^2$ will be displayed.

2.2.3. To obtain a preliminary $R_g$ use the "AUTORG" function, which is an external module built into PRIMUS/qt. Find the command prompt window and enter "autorg [datafile]".

2.2.4. Input multiple files at once by entering the same command prompt, but putting a space between each datafile name or by inputting all files in the selected folder by typing "autorg *.dat" which inputs every file ending in .dat.

2.2.5. Use the Guinier plot created earlier to assess the data quality; the green line under the Guinier plot shows the residuals plot representing a linearity of the fit. Be aware that non-linearity in the Guinier analysis could be a sign of sample aggregation and further analysis should not be performed in this case.

Note: A linear Guinier fit gives an $R_g$ with a small error (<5%) and suggests a high-quality sample.

2.3. Kratky Analysis

2.3.1. Load the data to be visualized in a similar manner as described above.

2.3.2. Click on the “SELECT” next to the data file name followed by “PLOT”. This will plot the data in a separate window.

2.3.3. Click the “SASPLOT” button below the “PLOT” button.

2.3.4. Click “VIEW” and subsequently select “Y*s^2::X” which will plot the data as “$q^2$ x L(q) vs $q$”. Be aware that globular proteins display a Gaussian peak, while unfolded proteins will display a plateau instead of a peak and resemble a hyperbolic plot[17].

2.4. Data Merging

2.4.1. Load buffer subtracted data for each concentration in PRIMUS/qt once again, as in step 2.1.1.

2.4.2. To merge the data, simply click on the “MERGE” button in the processing window.

2.4.3. Inspect each curve and the I Scale number, which correlates to the dilutions made from the original sample.

Note: Samples at higher concentration display less noise in the tail region of the curves.

2.5. P(r) Distribution

2.5.1. To generate the P(r) plot, load the merged data curves into PRIMUS/qt as previously described.

2.5.2. Load the “GNOM” module by typing “GNOM” into the command prompt area.

2.5.3. Click on the “GNOMPLOT” button to open a new window presenting the merged data of intensity of scattered light *vs.* $q$ and pair-distance distribution function plot on the right-hand side.

Note: The information on the right-hand side presents the overall quality of the pair-distance distribution function calculations.

2.5.4. Adjust the data range of the merged data to avoid any significant noise at the tail end of the raw data.

2.5.5. Omit data points close to the beam stop in the low-q region.

2.5.6. To determine the $D_{max}$, start with a range of ~5 times the $R_g$ obtained from the Guinier analysis. Gradually decrease this value until the P(r) plot does not abruptly drop to zero on the Y-axis and does not have a long-tail before approaching zero.

2.5.7. Check that the Experimental $R_g/I_0$ (derived from Guinier approximation) and $P(r)$ $R_g/I_0$ numbers are similar.

Note: In some cases, further manipulation on the range of data, data points, and ALPHA (a regularization parameter which tells the program the ratio of how much attention is paid to the smoothness of the distribution compared to fitting the experimental data) is also required[21] to obtain a good quality *P*(r) plot.

**3. A*b initio* bead modeling and averaging**

3.1. Once the data collected at multiple concentrations are merged, or the data collected using S-SAXS is minimized, and the Kratky plot, $P(r)$ plot and Guinier analysis have been verified, calculate low-resolution structures of macromolecules and their complexes. This pipeline can be used to study solution structures and interactions of nucleic acids, proteins, and nucleic acids-protein or protein-protein complexes[10,11,21–34]. One of the most popular programs is DAMMIN, developed by Svergun[35] and a part of the ATSAS package[13], which employs simulated annealing protocols with preliminary input information on $R_g$ and $D_{max}$. The *ab initio* modeling approaches and principles are described in detail elsewhere[18,36].

**REPRESENTATIVE RESULTS:**

The data analysis approach described above was utilized to calculate the $R_g$ and $D_{max}$ for nidogen-1, laminin γ-1, and their complex using the $P(r)$ function. We obtained $R_g$ values of 7.20 (±0.10) nm, 8.10 (±0.20) nm, and 10.9 (±0.4) nm for nidogen-1, laminin γ-1 and their complex respectively (**Figure 2A-B**). In addition, $D_{max}$ values of 24 nm, 26 nm, and 35 nm for nidogen-1, laminin γ-1, and their complex respectively (**Figure 2**)[10] were obtained. The DAMMIF program was used to obtain low-resolution structures of nidogen-1 and laminin γ-1, which suggested that both proteins adopt an extended shape in solution. The *X* and NSD values for nidogen-1 (~1 and 0.8) and laminin γ-1 (~0.9 and 0.8 respectively) were also in the acceptable range. The alignment of high-resolution structures, two domains of nidogen-1 and two of laminin γ-1, on their low-resolution structures obtained using SAXS allowed identification of their N- and C-terminal regions[10].

Nidogen-1 was identified as an interacting partner of laminin γ-1[39,40] and the interaction site was mapped using X-ray crystallography to the C-terminal domains[41]. However, high-resolution structures only involved interacting domains and not the full-length nidogen-1 or the entire laminin γ-1 arm. Therefore, we purified a complex containing nidogen-1 (full length) and the laminin γ-1 arm to identify the interacting regions as well as to study the relative orientation of the N-terminal domains of both proteins. The SAXS data for the complex yielded an $R_g$ of 10.9 (±0.4) nm and a $D_{max}$ of 35 nm. We utilized MONSA to obtain the low-resolution structure of the

entire complex, which suggested that indeed, only the C-terminal region of both proteins participate in mediating interactions, whereas the rest of the domains are far apart from each other (**Figure 3**, **Video 1**).

**FIGURE AND TABLE LEGENDS:**

**Figure 1.** Schematics of SAXS set-up. A monodispersed preparation of biomolecules or their complexes is prepared, followed by exposure with high energy X-rays. Depending on the source (*e.g.*, in-house *vs.* synchrotron), the energy of X-rays and the sample to source distance can vary. The X-rays' scattering pattern (that depends on the size and shape of biomolecules) is recorded and radially averaged to obtain a 1-dimensional plot (1D) that contains information on the intensity of scattered light with respect to the scattering angle. As buffer molecules also scatter light, the contributions from these molecules are subtracted to obtain a scattering pattern of the biomolecules of interest. At the synchrotron, prior to the SAXS data collection, an additional purification step using in-line size exclusion/high-performance chromatography is also typically performed (top view). This step is critical to remove any aggregated and/or degraded product as well as to remove any unbound biomolecules from the complex. The 1D scattering plot is converted to the electron pair-distance distribution plot (*P*(*r*) plot), which provides the radius of gyration and maximum particle dimension of biomolecules. This plot is used as the input file for the *ab initio* modeling packages (*i.e.,* DAMMIN/DAMMIF) to obtain low-resolution structures of biomolecules, or other packages (*i.e.,* SASREF/CORAL) if the high-resolution structure of parts of the biomolecules or individual biomolecules of the complex is known.

**Figure 2.** (A) A plot of an intensity of scattered light *vs.* scattering angle ($q=4\pi\sin\theta/\lambda$, $nm^{-1}$) suggesting the quality of biomolecules (low region) and shape (high region) of biomolecules. (B) The electron pair-distance distribution *P*(*r*) determined from the scattering data suggest an elongated shape of biomolecules under investigation (laminin γ-1, nidogen-1, and their complex). (C) Kratky plot suggesting that nidogen-1 and laminin γ-1 proteins are not unfolded. (D) Guinier plot for nidogen-1, laminin γ-1 and their complex, indicating the linear region for determination of the radius of gyration using data at low-scattering angle.

**Figure 3.** Low-resolution structure of the complex of nidogen-1, and laminin γ-1 obtained by analysis of merged data sets using the program MONSA. The color scheme is the same as **Figure 2**.

**Video 1**. The low-resolution structure of the nidogen-1 and laminin γ-1 complex. This movie was prepared using PYMOL to visualize various structural features of the complex. The crystal structure of the laminin-nidogen complex (PDB ID: 1NPE) is shown as ribbon cartoons, highlighting the interacting sites for this complex. The color scheme is the same as **Figure 2**.

**DISCUSSION:**

The critical steps of SAXS data analysis outlined in the protocol section of this paper include buffer subtraction, Guinier analysis, Kratky analysis, data merging and P(r) distribution. The *ab initio* bead modeling is too extensive to be covered here in detail and is therefore only covered briefly.

At synchrotrons (*e.g.* DESY in Germany, DIAMOND in the UK and ESRF in France), it is possible to collect SAXS data for a very tiny fraction (~few µL) of each sample as the fractions are being eluted from the s column that is connected in-line (see **Figure 1**). The elastically scattered SAXS data is radially averaged using the packages provided by the instrument manufacturer or by the synchrotron before buffer subtraction can take place. The resulting 1D data represents the amount of scattered light (In $I(q)$) on the $Y$-axis and scattering angle ($q$=4πsinθ/λ, where λ is the wavelength of incident $X$-rays) and is outlined in **Figure 1**. The program PRIMUS/qt[12] is used to directly subtract any background due to buffer and is described in section 1.1. Other programs such as; ScÅtter[43] (download available at www.bioisis.net) with a tutorial available at https://www.youtube.com/channel/UCvFatdC5HcZOLv6OSjblfeA, and bioXtas RAW[44] (available at https://bioxtas-raw.readthedocs.io/en/Latest/index.html) can be utilized as an alternative to the ATSAS package.

The Guinier analysis provides information on sample aggregation and homogeneity as well as providing the Radius of Gyration ($R_g$) for the macromolecule of interest based on the SAXS data from the low $s$ region[14]. A plot is constructed with PRIMUS/qt for SAXS data obtained from each concentration, followed by curve fitting with the maximum range of up to 1.30 for $q$ x $R_g$. A monodispersed sample preparation should provide a linear Guinier plot in this region (**Figure 2D**), whereas aggregation results in a nonlinear Guinier plot[15,16]. If the Guinier analysis is linear, the degree of "unfoldedness" of a macromolecule of interest can be observed with the Kratky plot, which is useful when deciding whether to perform rigid body modeling or construct ensembles of low-resolution models. A globular protein will appear in a Kratky plot to have a bell-shaped curve, whereas extended molecules or unfolded peptides will appear to plateau or even increase in the larger $q$ range and lack the bell-shape (**Figure 2C**).

Obtaining the $R_g$ from Guinier analysis only considers data points from the low $q$ region of the 1D scatter plot (**Figure 2D**), however, it is possible to use almost the entire dataset to perform an indirect Fourier transformation to convert the reciprocal-space information of ln(I($q$)) *vs.* ($q$) into a real space distance distribution function ($P(r)$) which provides information on $D_{max}$ and $R_g$ (**Figure 2B**) The shape of the $P(r)$ plot represents the gross solution conformation of the macromolecule of interest[18,19]. the conversion of reciprocal-space data to real-space data is a critical step but a detailed description is not within the scope of this paper. Therefore, refer to an article by Svergun[20] to understand each parameter.

Once the buffer subtracted data at individual concentrations are processed through Guinier analysis with a consistent value for $R_g$, followed by investigating their folding pattern using Kratky analysis, these data can be merged. The merged data for nidogen-1, laminin γ-1, and their complex were processed as described above and the resulting P(r) plots are presented in **Figure 2B**. Ideally, one should also calculate the pair-distance distribution function P(r) for each concentration to determine if SAXS data collected for each concentration provides similar $R_g$ and

$D_{max}$ values. If the $R_g$ and $D_{max}$ remain similar over a wide range of concentrations, then the user should proceed. It should be noted that depending on the signal, data can be truncated prior to data merging. This is often the case if the concentrations and/or molecular weight of the macromolecules under investigation is low.

Low-resolution shape analysis using DAMMIN can be performed in various modes (*e.g.* Fast, Slow, Expert modes, *etc.*). The Fast mode is an ideal first step to evaluate if the P(r) plot provides good quality models. Typically, at least 10 models should be obtained for each P(r) plot to check if reproducible results, in terms of the low-resolution structure, are obtained, with a low goodness of fit parameter called χ (a value of 0.5-1.0 is considered good based on our extensive work), a value that describes an agreement between experimentally collected SAXS data and model-derived data. For publication purpose, we typically use Slow or Expert mode and calculate at least 15 models. In addition to DAMMIN, a faster version of it, DAMMIF[37], as well as GASBOR[38] are also alternatives. Furthermore, to study protein-protein or protein-nucleic acid complexes, it is possible to use the MONSA program[35], which facilitates simultaneous fitting of the individual SAXS data for both macromolecules as well as their complex. For more details on high-resolution model calculations as well for RNA-protein interaction studies, refer to a recent article by Patel *et al*[3].

SAXS is theoretically simple but undoubtedly a highly complementary method to other structural biology tools and results in low-resolution structural data that can be used on its own or in conjunction with high-resolution techniques to elucidate information about macromolecular structure and dynamics. As long as a monodispersed preparation of macromolecules and their complexes can be obtained, SAXS can be utilized to study in-solution structure and interactions of any type of biological macromolecule. In the case of the complex discussed here, it is remarkable that less than 10% of the overall accessible surface area of nitrogen-1 and laminin γ-1 is buried in this complex, whereas the rest of the domains of both proteins are freely accessible to interact with other proteins at the extracellular matrix to maintain its structural rigidity (**Figure 3**). Obtaining such information for a complex with ~240kDa would be very challenging using other structural biology techniques such as X-Ray Crystallography, NMR, and Cryo-EM Microscopy.

Uncovering protein structure via X-Ray Crystallography or NMR is an inherently time-consuming process. This bottleneck in structure determination is one area where SAXS shows its strength as a structural technique; data acquisition for a single SAXS experiment can take less than an hour and with the help of streamlined analysis software, analysis can be done quickly and efficiently. SAXS has the potential to greatly increase throughput of structural studies as a stand-alone technique because it offers a low-resolution model of the macromolecular structure before high-resolution data is available. A barrier to other structural techniques is the requirement for a highly pure, concentrated sample for data acquisition, which necessitates a high level of protein expression and stability over a long period of time. While SAXS samples also need to be pure and concentrated, the sample volumes are roughly 100 µL making SAXS a relatively inexpensive method of analysis compared to other structural techniques. Moreover, SAXS coupled with size exclusion chromatography is becoming increasingly common which provides an additional quality control step. Recently there has been strong advances in the combination of NMR and

SAXS data using the Ensemble Optimization Method (EOM)[45,46] to elucidate flexible systems. In a recent paper by Mertens and Svergun[47], the authors describe multiple recent examples of EOM SAXS in combination with NMR, along with many other examples of SAXS data being used in conjunction with NMR. Advances are continually being made in the field of SAXS, and new techniques are being developed for SAXS to be used in conjunction with, not just complimentary to, other structural techniques. Consequently, we believe that the demand for SAXS will only increase over time, especially in conjunction with NMR to characterize dynamic systems where functions are defined by flexibility.

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**DISCLOSURES:**

Authors have no disclosures to declare.

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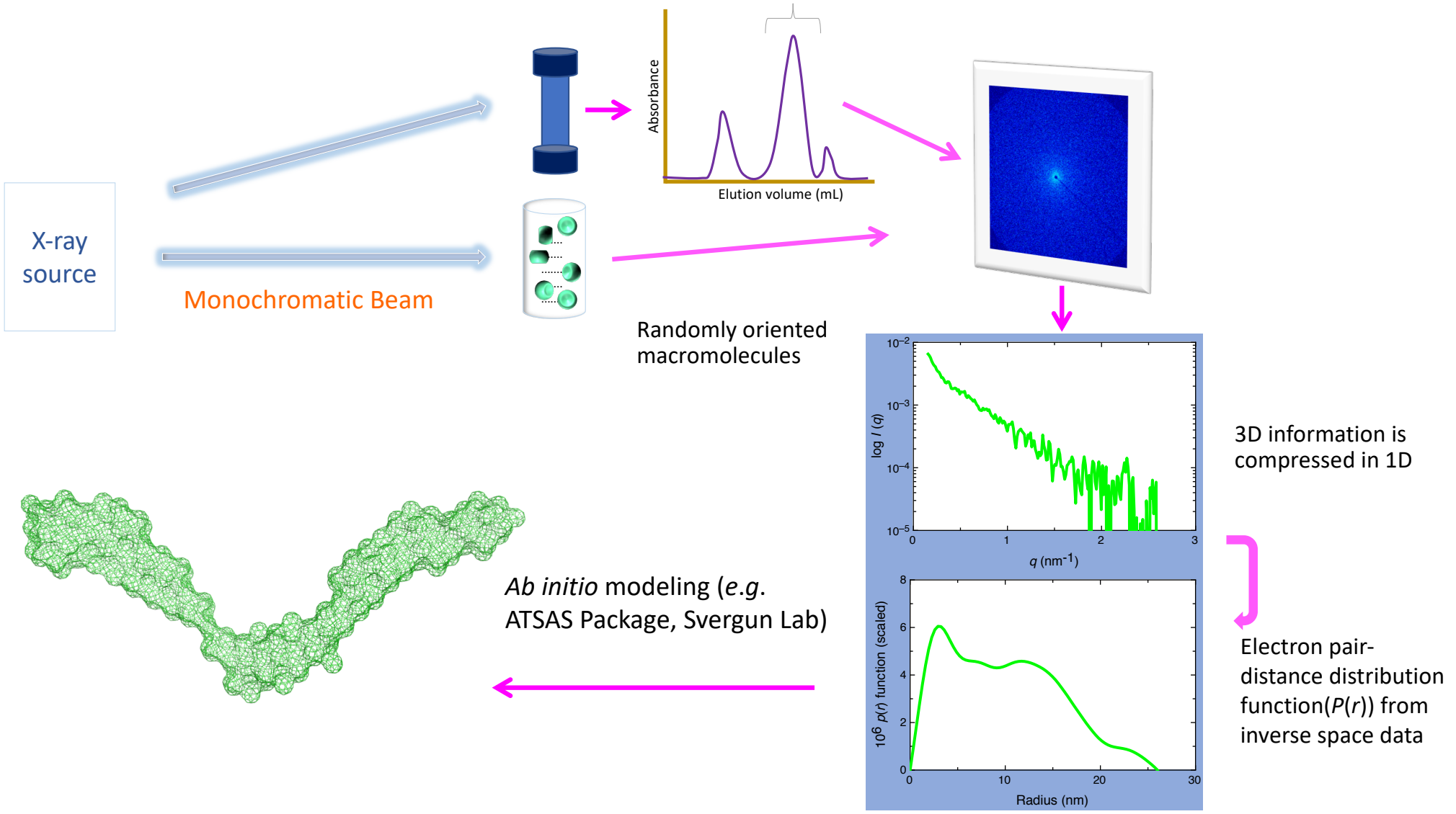
Figure 1





gure 2



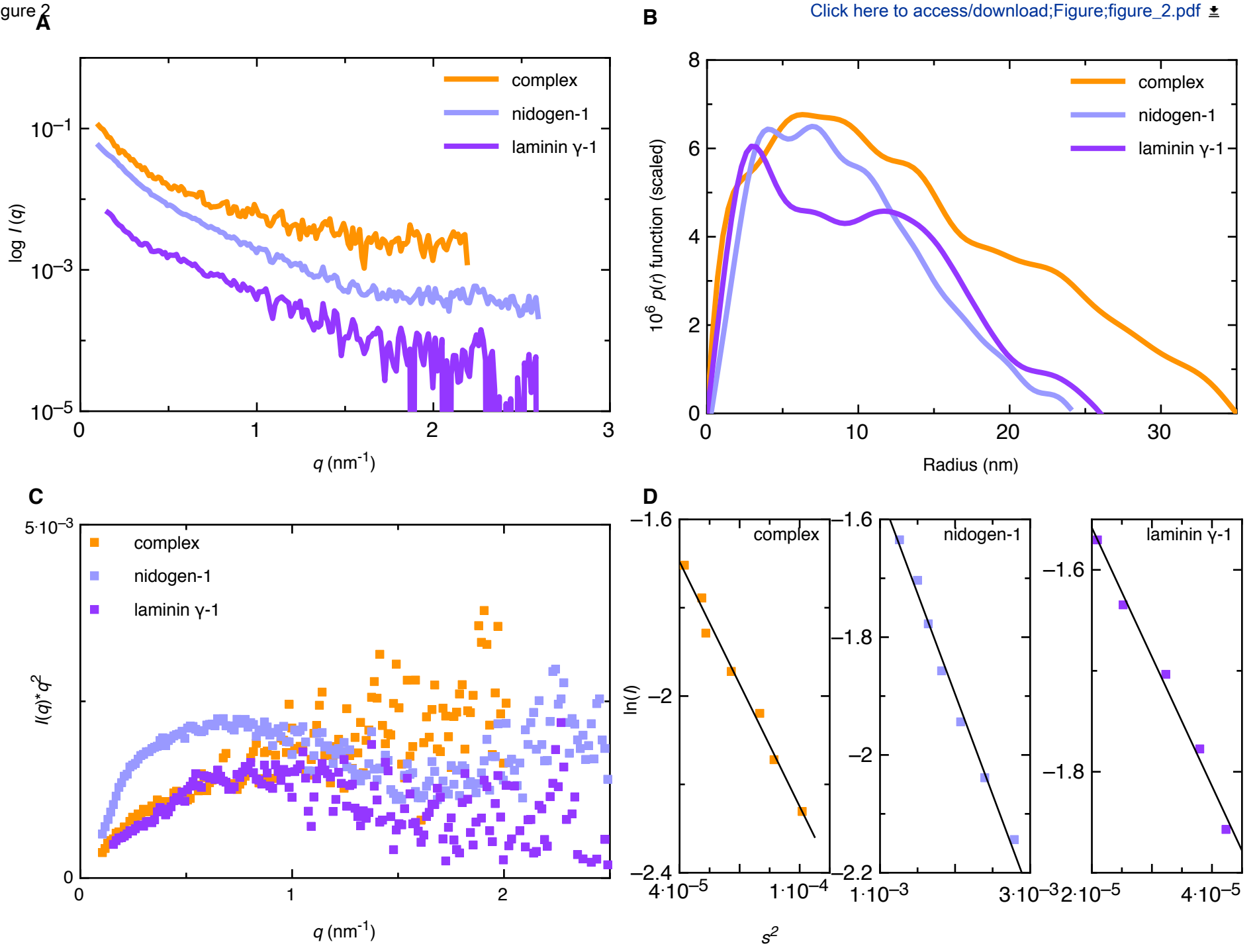
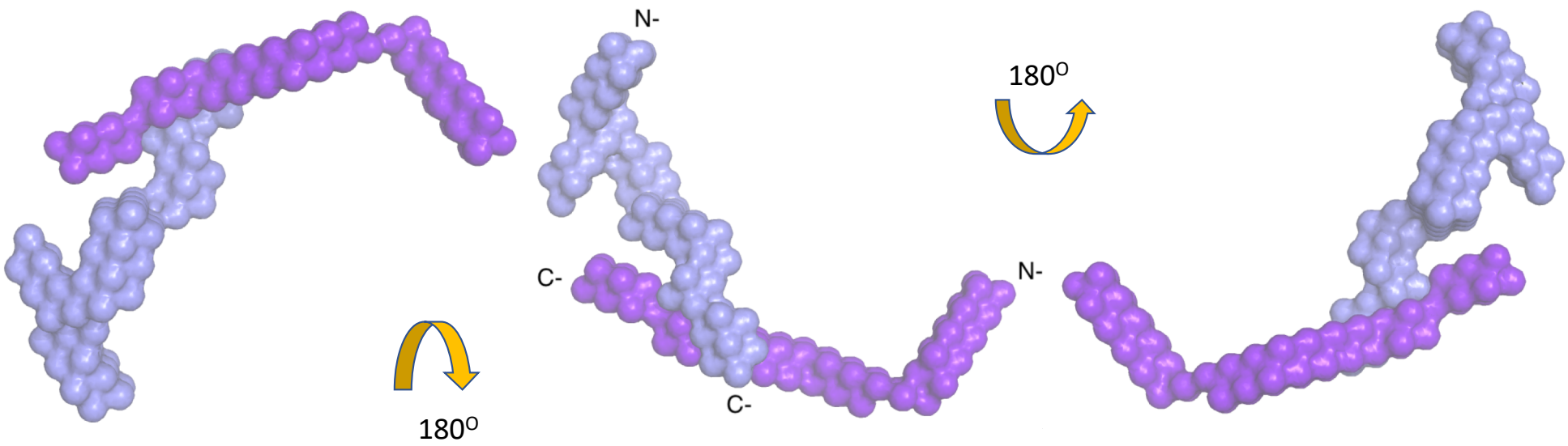


Figure 3





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| Name of Material/ Equipment | Company | Catalog Number |
|---|---|---|
| HEK 293 EBNA Cell Line | In-Lab availability | - |
| Ni Sepharose High Performance histidine- | GE Healthcare | 17524801 |
| Superdex 200 Increase 10/300 | GE Healthcare | 28990944 |
| ÄKTA Pure FPLC | GE Healthcare | - |
| Nanodrop | Nanodrop | - |
| Basic Reagents (NaCl, Tris-HCl etc.) | | |
| S-MAX3000 | Rigaku | - |
| Zetasizer Nano-S | Malvern Instruments Ltd | - |
| 0.1µm Filter | Millipore | JVWP04700 |
| Thrombin cleavage kit | abcam | ab207000 |
| Strep-Tactin Sepharose Column | IBA | 2-1201-010 |
| D-desthiobiotin | Sigma-Aldrich | 533-48-2 |
| **Software** | | |
| SAXGUI | Rigaku | - |
| ATSAS Suit | Franke et al., 2017 | - |
| PRIMUS | Konarev *et al.* , 2003 | - |
| GNOM | Svergun, 1992 | - |
| DAMMIF | Franke and Svergun, 2009 | - |
| DAMAVER | Volkov and Svergun, 2003 | - |
| MONSA | Svergun, 1999 | - |
| GASBOR | Svergun *et al* ., 2001 | - |
| DTS Software V6.20 | Malvern Instruments Ltd | - |
| PyMOL | Schrodinger, LLC. | - |
| The Protein Data Bank | Berman *et al* ., 2000 | - |

**Comments/Description**

Cell line used to overexpress protein(s)
Affinity protein purification resin
SEC Column
FPLC System
Spectrophotometer

SAXS Pinhole Camera System
Dynamic Light Scattering instrument
Used to Concentrate Sample Prior to DLS
Thrombin cleavage to remove His tag
Strep-Tag Affinity Purification
Elution of Strep Tag Protein

Data Collection for SAXS and data reduction
SAXS Data Analysis Software program suite
Buffer Subtraction
Rg, $D_{max}$ and p(r) Calculation
*Ab initio* model calculation
Averaged Solution Conformation calculations
Simultaneous model fitting for the complex
Alternative Ab initio model calculations
DLS supplied instrument software
The PyMOL Molecular Graphics System V2.0
PDB ID: 1NPE

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4. Please include a space between all numbers and their corresponding units: 15 mL, 37 °C, 60 s; etc.

*O We have ensured that there is a space between all numbers and their corresponding units.*

5. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. For example: Rigaku, S-MAX3000, MicroMax, Mac OS, YouTubeTM, etc.

*O We have ensured that no commercial trademark symbols are present in the manuscript.*

6. Please revise the protocol to contain only action items that direct the reader to do something (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” Please include all safety procedures and use of hoods, etc. Please move the discussion about the protocol to the Discussion.

*O We have revised this section and Discussion accordingly.*

7. Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

*O We have revised this section to include the changes recommended.*

8. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please simplify the Protocol so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step. Use sub-steps as necessary. Please move the discussion about the protocol to the Discussion.

*O We have revised this section to include the changes recommended.*

9. Please include single-line spaces between all paragraphs, headings, steps, etc.

*O We have implemented this suggestion throughout the manuscript.*

10. After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

*O The Protocol section is highlighted by the bold-font title – **PROTOCOL** in grey background*.

11. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense.

*O We have implemented this suggestion In Protocol section.*

12. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

13. Please number the figures in the sequence in which you refer to them in the manuscript text.

*O Figures are numbered according to their order of description in the text.*

14. As we are a methods journal, please revise the Discussion to explicitly cover the following in

detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique
15. Please remove trademark (™) and registered (®) symbols from the Table of Equipment and Materials.

*O We have implemented these suggestions in a revised Discussion section.*

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**Reviewers' comments:**

**Reviewer #1:**
Manuscript Summary:
In this manuscript the authors provide a protocol for using SAXS (small angle X-ray scattering) to determine hybrid structures of macromolecular assemblies. The authors provide a recent example of the complex of full length nidogen-1 and laminin which assembles into an extended curved shape. This manuscript is well written and provides a very nice introduction to SAXS including an overview of its benefits and limitations. This manuscript is acceptable for publication, however I have concerns about the current format of the protocol because it contains numerous lengthy paragraphs that should be broken down into smaller segments.

Major Concerns:
The protocol is written in paragraph format, which differs from the typical JoVE protocol format. The authors provide very detailed descriptions of each step in the SAXS data processing pathway but they combine everything into one paragraph and don't break things down into discrete steps. For example Section 3.4, should be broken down into individual steps as follows:
3.4.1 Load the GNOM module ….
3.4.2 Generate the p(R)plot …
3.4.3 Quality control check of the data …
3.4.4 Adjust the Dmax …

*O We have addressed reviewers' suggestions in a revised Protocol section.*

Minor Concerns:
1. SAXS Sample Prep: This section is not really part of the protocol but a comment since no specific details are included. The authors should either include specific details or make this a simple note about sample prep and not part of the protocol.

*O We have included the importance of sample preparation in **SAXS Sample Preparation and Data Acquisition** section.*

2. Data Acquisition: This section is also more of a comment and not part of the protocol. The authors should either include the details for specific data collection of the Lamin complex or format this as a comment/note and not the protocol.

*O We have revised **Protocol** section to implement reviewers' suggestion.*

**Reviewer #2:**
Manuscript Summary:
The JOVE manuscript from Mrozowich et al. "Structural Studies of Macromolecular Interactions in Solution using Small Angle X-Ray Scattering" seeks to provide a detailed protocol for scientists using SAXS as a tool in their research. The manuscript is well timed and with some additional work should achieve this aim.

However, a major concern is that the detailed description of the ATSAS software is very much out of date. The authors describe in detail how to use the original windows version of PRIMUS, which is no longer in common use (at the very least this is what the package maintainers say during their many workshops and recent publications). I feel that if the authors were to describe their protocol using the latest cross-platform version (primusQt for example) it would be a more useful and appropriate contribution. An alternative is to provide the analysis steps using another modern program that has been published and available such as bioXtas RAW (Hopkins, J.B., Gillilan, R.E. and Skou, S., 2017. BioXTAS RAW: improvements to a free open-source program for small-angle X-ray scattering data reduction and analysis. Journal of applied crystallography, 50(5), pp.1545-1553.; Nielsen, S.S., Toft, K.N., Snakenborg, D., Jeppesen, M.G., Jacobsen, J.K., Vestergaard, B., Kutter, J.P. and Arleth, L., 2009. BioXTAS RAW, a software program for high-throughput automated small-angle X-ray scattering data reduction and preliminary analysis. Journal of applied crystallography, 42(5), pp.959-964). The authors indicate that another program called Scatter can be used, however, there appears to be no publication in a peer reviewd journal of this software, so perhaps it is better to focus on ATSAS and RAW.

*O We have revised the **Protocol** section for PrimusQt version. Due to the constraints with space (~2.5 pages for **Protocol** section), we were unable to include the detailed description of other data analysis packages suggested by the reviewers. However, we recommend that subsequent protocol/methods papers should be published in the future describing the packages suggested by the reviewer.*

In addition, several
suggestions to improve the reader experience are:

1. Make it clear that the scattering phenomenon under discussion is elastic.

*O We have made this clear in the Discussion section.*

*2. Provide a better explanation of the determination of concentration dependence (not just suggest "the Rg of each data set should be compared to avoid interparticle interaction").*

*O We have briefly described this in the **Principles, Benefits, and Limitations of SAXS** section.*

3. In the SAXS Sample Preparation section: explain why particle size is an important parameter in the range of concentrations one should consider.

*O We have included this in **Principles, Benefits, and Limitations of SAXS** section.*

4. Ensure that a figure is made available of the Guinier analysis and also the Kratky plot, showing graphically what interparticle interaction and "foldedness" look like.

*O We have revised Figure 2 to include Guinier and Kratky plots.*

And finally, make sure that the aims of the manuscript as defined in the abstract are clearly met:

1. protocol is provided for elucidating the "specific domains" that mediate interactions. I don't think this is clear, a protocol for basic analysis is provided but I do not it currently addresses this specific aim sufficiently.

*O We have revised sections of the manuscript to address this change.*

2. method is provided for calculating hybrid solution structures... I do not think it is currently clear to the reader that what is described provides a method for doing this. A little work is required to address this.

*O We have revised sections of the manuscript to highlight that SAXS can be used to study macromolecules and their complexes.*

3. "allows large numbers of samples to be analysezed..." This I don't think is addressed at all.

*O We have revised this sentence in Abstract.*

**Reviewer #3:**
Manuscript Summary:
This manuscript reviews the method of SAXS to study macromolecules in solution. When combined with other high resolution structural methods, SAXS analysis provides important insights into the mechanisms of macromolecular interactions. The manuscript has reviewed the basic and essential methods, such as Guinier, P(r) analyses, as well as the ab initio methods to

restore molecular shapes using DAMMIN and Monsa. The manuscript is certainly worthy to be published.

Major Concerns:
The authors might also discuss some of the recent progress in ensemble analysis of SAXS data, which is quite useful when combining with NMR methods.

*O We have included a paragraph in the Discussion section for this. We believe that the combination of SAXS and NMR for ensemble analysis merit a separate publication to provide an in-depth description of benefits of these two powerful tools.*

Minor Concerns:
Some of the sentences in the text are long, and awkward to read.

*O We have revised sentences to ensure that they are not too long and awkward to read.*