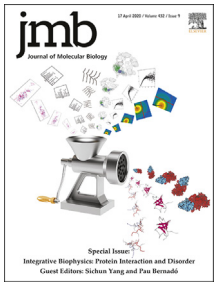
Review



Integrative/Hybrid Modeling Approaches for Studying Biomolecules

Ashutosh Srivastava [1], Sandhya Premnath Tiwari [2], Osamu Miyashita [2] and Florence Tama [1,2,3]

*1 - **Institute of Transformative Bio-Molecules,** Nagoya University, Nagoya, 464-8601, Japan*
*2 - **RIKEN-Center for Computational Science,** Kobe, Hyogo, 650-0047, Japan*
*3 - **Department of Physics,** Graduate School of Science, Nagoya University, Nagoya, 464-8602, Japan*

***Correspondence to Florence Tama:** Nagoya University, 1 Furo-cho, Chikusa-ku, Nagoya, 464-8601, Japan.*
florence.tama@nagoya-u.jp.
https://doi.org/10.1016/j.jmb.2020.01.039
Edited by Sichun Yang

Abstract

The structural and dynamical characterization of biomolecules holds central importance in the endeavor to understand the molecular mechanisms regulating living systems. However, owing to the inherent heterogeneity of biomolecular interactions within cells, it is often difficult to understand the overall structure and dynamics of biomolecules using any experimental method in isolation. In this regard, hybrid methods that combine data from multiple experiments to generate a comprehensive model of biomolecular complexes have gained prominence in the last few years. In this article, we discuss the advancements in hybrid methods, with a particular focus on the role of computation in their development and application. We further outline the future directions that hybrid methods are likely to take, regarding the advancements in techniques such as X-ray free-electron laser single- particle imaging, and electron cryo-tomography. Finally, we conclude the review by highlighting the future goals of broader consensus and collaboration within the integrative/hybrid structural biology community and for disseminating the data generated by hybrid modeling efforts.

Introduction

For a long time now, several seemingly diverse fields have contributed toward development of techniques to explore the atomic structure of biomolecules. The renowned physicist and Nobel Laureate Richard Feynman once said in his popular lecture series.

"… if we were to name the most powerful assumption of all, which leads one on and on in an attempt to understand life, it is that *all things are made of atoms*, and that everything that living things do can be understood in terms of the jigglings and wigglings of atoms." (http://www.feynmanlectures.caltech.edu/I_03.html).

By characterizing the three-dimensional (3D) atomic structure of biomolecules, we come closer to observing the "jigglings and wigglings" of their constituent atoms, allowing us to better understand living systems.

The theories of diffraction and Bragg's law with mathematical concepts of Fourier transform paved way for the determination of the first protein structure [1]. Similarly, the development of NMR as a technique for structural and dynamical studies of macromolecules required great efforts not only in the development of NMR instrumentation but also in the labelling techniques and data analysis [2–4]. More recently, the breakthrough in determining high-resolution structures through cryo-electron microscopy (cryo-EM) can be greatly attributed to the immense developments in detector technology and the exponential increase of computational power with concurrent algorithm and software development [5,6]. In addition, information on dynamics are essential to elucidate the mechanism of biomolecular functions and a variety of time-resolved experiments are being developed. For example, time-resolved spectroscopy [7] as well as solution

scattering [8] have been used to study the conformational dynamics of a variety of proteins. Furthermore, recent advances in time-resolved crystallography methods, particularly using X-ray free-electron laser (XFEL) pulses, pave the way toward high-resolution characterization of dynamics in challenging systems such as membrane proteins and capturing the enzyme catalysis [9–14].

Concomitant to the development of experimental techniques for structure determination, computational approaches to interpret and analyze the structural data have been developed steadily by the structural biology community [15]. Experimental approaches have their limitations when trying to understand the structure and dynamics of biomolecules [16], some of which can be mitigated by combining multiple experimental approaches. Although, integrative or hybrid methods that combine different experimental approaches, have been utilized for quite some time to explore macromolecular structures, their use has seen tremendous growth in last few years [17]. Not only is the application of integrative methods becoming commonplace to study macromolecular structure, the data generated in the form of integrative models are becoming available for larger use and dissemination [18,19]. The number of integrative models has been steadily increasing over the years. As of September 2019, 23 integrative models have been deposited in the PDB Dev database, out of which 6 have been deposited in 2019, and the oldest structure was published in 2014. This number by no means reflects the actual number of integrative modeling studies that have been carried out over the years. Several factors have contributed to this spurt in integrative structural biology, of which we will discuss some of the most crucial developments.

The structures, determined using cryo-EM, have seen almost an exponential growth in past few years, with vast improvements in resolution [20,21]. Among these, the share of EM maps with near atomic resolution ($\leq$4 Å) has increased drastically from ~28% in 2017 to ~49% in 2019 (https://www.ebi.ac.uk/pdbe/emdb/statistics_main.html/). This revolution in cryo-EM has brought biological systems that were previously difficult to characterize, such as membrane proteins and large complexes, within the scope of study [22]. However, a majority of cryo-EM models still do not provide atomic resolution biomolecular structure. This is further complicated by the fact that the reported resolution of EM maps might not reflect a higher level of detail in their structural features and generally show poor agreement with corresponding atomic models [23,24]. Therefore, the development of other experimental approaches to derive atomic level understanding has been crucial.

One such method that stands out as a crucial factor in the development of integrative structural biology, particularly for modeling of macromolecular complexes, is cross-linking mass spectrometry (XL-MS) [25]. Owing to advances in experimental protocols, better understanding of cross-linking chemistry and the development of better cross-linking agents and detection techniques, XL-MS has become a quintessential part of integrative structural biology [26]. Another EM-based method that has recently gained prominence in determining the high-resolution structures of proteins that are difficult to crystallize is micro-electron diffraction (micro-ED). Micro-ED capitalizes on the advancements made in the field of cryo-EM and X-ray crystallography by combining both techniques. A high-resolution structure is determined by capturing electron diffraction patterns from protein crystals that range between nanometer to micrometer in size [27,28].

The growth in integrative structural biology also owes a great deal to the unprecedented advancements in computational capabilities over the years. The data generated from multiple experiments require not only interpretation but also reconciliation over a wide range of resolution and time scales. This has led to the development of algorithms that integrate diverse types of data from different experimental studies.

In this review, we will explore the role of computation in the development of hybrid methods for integrative structural biology, followed by some of the recent examples of implementations that provided insight into specific questions regarding structural biology. Finally, we will review the new frontiers in the field of integrative/hybrid structural biology and modeling.

Role of Computation in Hybrid Methods

Hybrid methods aim to integrate data coming from multiple sources [29]. Computationally, this translates to interpreting data coming from biophysical and biochemical experiments, extracting relevant structural information and generating structural models that best explain the experimental data. The biggest challenge in this is to construct a model that is a faithful representation of the experimental data without overfitting. Conventionally, when performing integrative modeling, some structural information is usually available, as a crystal or NMR structure of the whole or part of the complex under investigation. The structural information can also be derived using homology modeling in cases where one or more template structures are available, *ab initio* modeling in cases where there is no known related model, and coarse-grained modeling [30,31]. The efforts in protein structure prediction have recently been invigorated by utilization of deep learning methods and co-evolutionary analysis [32]. Simultaneously, low-resolution experimental data are being increasingly used to generate more

accurate protein structure models as was observed in the recent rounds of the critical assessment of protein structure prediction (CASP) experiments [33–35]. In terms of generating an integrative model, there are two major components of hybrid computational methods:

1) Sampling or conformational search: Dynamics of macromolecules play a crucial role in defining function not only as individual units but also as part of larger complexes. A direct consequence of this, in terms of experimental and computational structural analysis, is that often, the macromolecular conformations observed using different experimental techniques vary according to environment, time scale and functional state. Thus, for reconciling biophysical and biochemical data coming from multiple sources, at varying resolutions, and different time scales, computation plays a crucial role by providing various tools for searching through multiple conformations. Depending on the available structural information, the conformational search can be performed at a coarse-grained or a detailed atomic level. Some of the most commonly used methods for conformational sampling have been described in brief below. The details of these methods are beyond the scope of this review. A recent excellent review has discussed conformational sampling methods in detail [15].

   a. Molecular dynamics (MD) simulations: This is one of the most commonly used methods to explore the conformational dynamics of macromolecules. The motions associated with each atom are mapped as per classical mechanics approach of Newton's equation of motion in a time-dependent manner [36,37]. Performed for the first time for a biomolecule more than 40 years ago, MD simulations are now a routine method used to understand the dynamics of biomolecules. The two major challenges for MD simulations, that of achieving biologically relevant time scales of motions and of exploring large macromolecular systems, seem to be within reach of being resolved [38,39]. This is due to the development of both, better hardware, such as Graphic Processing Units and dedicated supercomputers [40,41], as well as software [42–44]. Additionally, several variations of MD simulations have also been developed over time to enhance the sampling of conformations, such as Replica Exchange Molecular Dynamics [45], Metadynamics [46], Parallel Cascade Selection Molecular Dynamics (PaCS-MD) [47], and Simulated Annealing [48]. In terms of hybrid modeling, MD simulations have been extensively used to fit the atomic or coarse-grained models of biomolecules to the low-resolution EM maps [49–54]. MD simulations have also been used to either generate models or understand the conformational dynamics of biomolecules when combined with scattering (SAXS and SANS) or fluorescence data (smFRET) [55–57].
   b. Monte-Carlo (MC) sampling: MC sampling is another commonly used method to generate diverse conformations to derive models satisfying experimental data during integrative modeling. MC sampling does not use Newton's equation of motion to calculate the subsequent position of the atoms of the biomolecule. However, it does use molecular mechanics-based force fields to calculate the potential energy associated with conformations that are selected based on the Metropolis criterion [58]. Like MD simulations, MC methods have also been used to generate integrative models when combined with low-resolution data from EM and SAXS [59–61]. MC methods have been particularly successful in determining the assemblies of subunits in low-resolution maps of large protein complexes [62,63].
   c. Normal mode analysis (NMA): NMA is a method used to characterize the vibrational properties, or intrinsic dynamics, of biomolecules in a time-independent manner [64–68]. Derived from a total potential function, normal modes describe energetically independent contributions to the potential energy of a given biomolecule, assuming that the initial position of its atoms are close to an energetic minimum. The large-amplitude *slow* normal modes from both all-atom and coarse-grained NMA have been shown to describe biologically relevant collective motions [69]. NMA has also been used in flexible-fitting for cryo-EM data [70–73], one-dimensional data [74] and as a means to explore conformational heterogeneity in EM data [75,76].

2) Implementation of restraints: With about 97% 3D structures in Protein Data Bank contributed by X-ray crystallography and NMR, these techniques remain the most

crucial source of high-resolution biomolecular structures. However, for the integrative modeling of large molecular complexes, structural information is also obtained from other biophysical methods such as small-angle X-ray scattering (SAXS), small-angle neutron scattering (SANS), Förster Resonance Energy Transfer (FRET), and XL-MS. In most cases, the structural information obtained from these methods can be converted into spatial restraints that can be used in many ways when generating structural models. SAXS profiles provide information about the overall shape of the biomolecule in solution along with the pair distribution function and radius of gyration [77]. Using multiple donor-acceptor pairs in single-molecule FRET (smFRET) experiments, several distance measurements can be made which can be used as restraints. Different computational strategies have been employed to use these one-dimensional profiles to model the 3D structure of macromolecules [60,74,78–81]. XL-MS provides information regarding spatial proximity of residues from different subunits of multimeric complexes [26]. The chemical crosslinks bind to spatially close reactive groups on different regions of biomolecular complexes, thus providing distance restraints that can be used in structure modeling steps to generate 3D models [82]. Restraints can also be obtained from biochemical and cellular experiments such as yeast two-hybrid, co-immunoprecipitation, surface plasmon resonance, alanine-scanning and other mutational experiments. The restraints obtained using various experiments can be used primarily in two roles;

a. Modeling subunit assembly and conformational change: Restraints can be used to refine an existing model for the whole macromolecule. In this respect, low-resolution EM maps and SAXS profiles have been used for a long time to fit existing atomic models, to understand the conformational changes and different states of the biomolecules captured under non-crystalline conditions in the lower-resolution data. Crucial insights have been obtained from existing atomic models by using low-resolution data and computational fitting methods [50,83–86]. As described in the previous section, conformational search is an integral part of hybrid modeling. A global search of the entire configurational space can be computationally challenging. Restraints obtained by experiments can limit the search space considerably, thus accelerating the modeling process. Depending on the system size, tens to hundreds of cross-links between subunits can be sufficient to obtain unique models [87,88]. Restraints obtained using yeast two-hybrid and co-immunoprecipitation also help in limiting the possible subunit arrangements in large multi-subunit complexes [89].

b. Validation of models: One of the most important aspects of the integrative approach is the validation of the structural model obtained using hybrid methods [87]. A subset of experimentally acquired restraints can be set aside to be used for the independent validation of the model. Computer-generated models which satisfy these specially reserved independent restraints are considered to be robust, thus increasing the confidence in the modeling process.

Application of Hybrid Methods to Understand Difficult Biological Problems

There are numerous examples of integrative modeling, many of which have been discussed previously, and readers are suggested to peruse these extensive reviews [17,87]. The determination of the architecture and subunit assembly of the multimegadalton protein machine, Nuclear Pore Complex, remains one of the foremost examples of Integrative or Hybrid modeling [63]. In this review, we discuss the most recent examples that portray the strengths of integrative modeling particularly for problems that are almost intractable by using any experimental technique individually.

Hybrid methods for large macromolecular assemblies

Cells are comprised of a multitude of large molecular machines that assemble in a coordinated manner to perform crucial functions [90]. These complexes are often dynamic, showing heterogeneity both in the composition and the conformation of constituent subunits. This makes their structural exploration a challenging task, not only with respect to the expression, purification *etc.*, but also in terms of biophysical techniques that can be utilized to

study these complexes. Integrating multiple experimental methods and judicious use of computation has helped in understanding many of these complexes whereas several more are on the verge of being explored, revealing crucial biomolecular mechanisms [17,87]. An example is the study on the function of an evolutionarily conserved protein complex, BBSome. Several mutations associated with ciliopathy Bardet–Biedl syndrome can be traced to the subunits of BBSome complex. The cryo-EM map of this huge complex, comprising eight subunits made up of 29 different domains, was obtained at 4.9 Å resolution [89]. However, even at this resolution, unambiguous placement of subunits proved to be difficult. It was only by using an integrative modeling approach combining data from multiple experiments, and computational modeling, that the molecular architecture of the complex could be determined. Experiments such as co-immunoprecipitation, yeast two-hybrid and XL-MS provided restraints to limit the search space for computational docking using Rosetta software suite [91]. Even a low-resolution Cα model provided crucial insight about function and potential conformational change

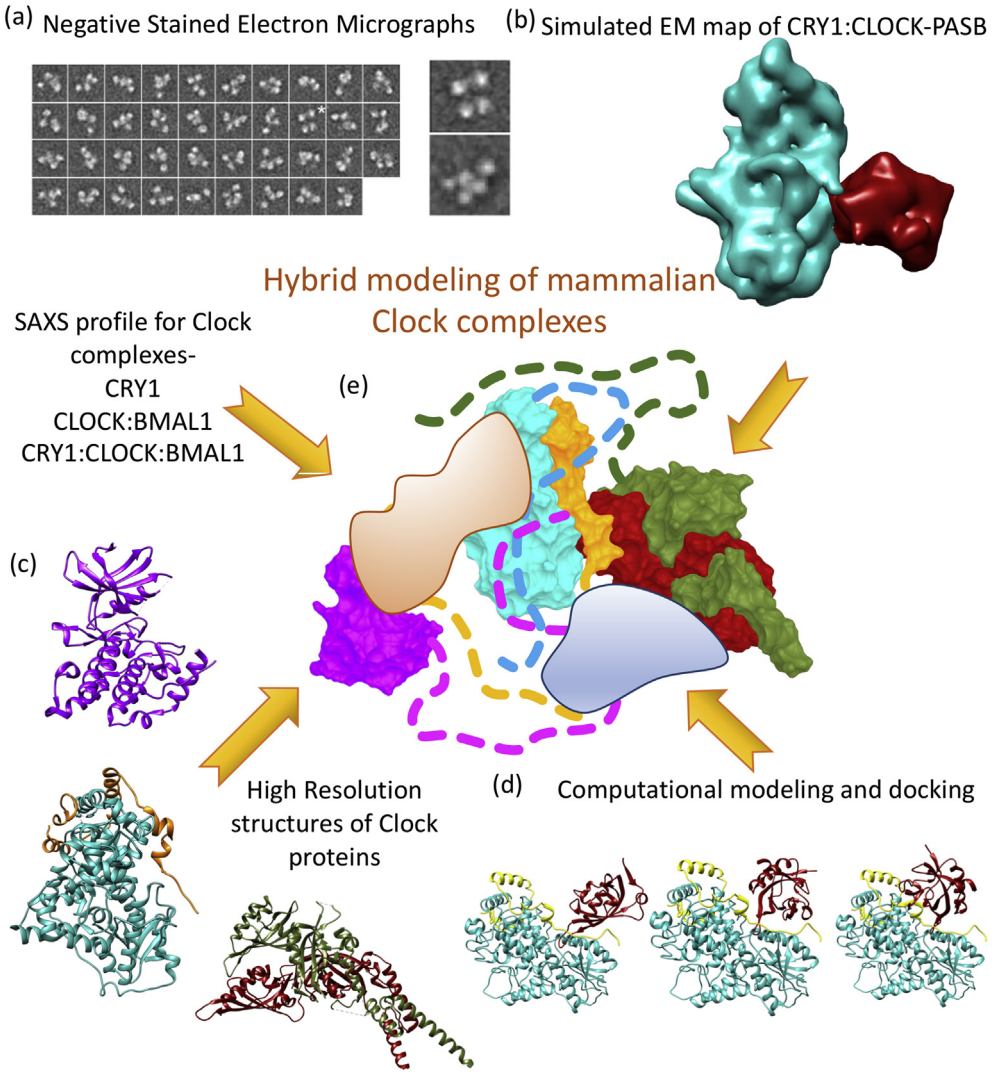


Fig. 1. Hybrid modeling of mammalian clock complexes. (a) Class averages of negatively stained cytoplasmic PER complexes [92]. SAXS for individual, dimeric and trimeric clock complexes have been determined [93]. (b) Simulated map of CRY1:PER2:CLOCK-PASB complex obtained from Haddock docking models and verified using experimental 2d back projections [94]. (c) High resolution crystal structures of components of mammalian clock complex- CK1δ [PDB ID: 6F1W], CRY1:PER2 [94], CLOCK:BMAL1 [PDB ID: 4F3I]. (d) Representative docking models from top three clusters of CRY1:CLOCK-PASB docking obtained by using HADDOCK, superimposed on the CRY1:PER2 crystal structure [94]. (e) Schematic representation of mammalian clock complex. Disordered regions have been represented by broken lines.

associated with the protein–protein interaction in this complex [89].

Another complex phenomenon that involves macromolecular assemblies of proteins within cells, but has lagged behind in structural exploration, is the molecular regulation of mammalian circadian rhythm. Physiology and behavior of almost all life forms is synchronized to a 24-h solar cycle called the circadian rhythm. In case of mammals, the intricate molecular mechanism controlling rhythmic expression of genes within a cell involves multiple dedicated clock proteins, kinases, phosphatases and transcription factors. Considering that protein complexes involved in this process can reach up to megadaltons in size, are highly dynamic in nature and contain a high percentage of disordered regions [92], structural characterization of these complexes requires an integrative approach (Fig. 1).

Toward the first step in resolving the multimeric complexes involved in mammalian circadian regulation, a model of the ternary complex between CRY1:CLOCK:BMAL1 was proposed using computational docking guided by mutational studies and SAXS [93]. This study revealed dynamic nature of the CLOCK:BMAL1 heterodimer and conformational change during interaction with CRY1 protein. Consequently, the cryo-EM map of this complex could only be obtained at intermediate resolution [94]. However, using hybrid methodology combining molecular dynamics simulations, docking and flexible fitting, a model of interaction between clock proteins CRY1 and PER2 with CLOCK PASB domain was determined, shedding crucial insight into role of dynamics at their interface in regulating circadian period [94]. Further investigation in this regard is underway and will require inputs from multiple sources.

Retrieving shape information from a few 2D EM images

For large native, dynamic complexes, as observed using negative stained images by Aryal et al. [92], it is quite difficult to perform 3D reconstruction. In such cases, it is useful to be able to draw meaningful conclusions regarding the overall shape of the complexes based on a single or few particle images. Recently Tiwari et al. assembled a non-redundant database of known structural models from the Electron Microscopy Databank, which were scaled to have the same size, to generate a library of 2D projection images for their matching protocol [95]. In their method, a few input images (e.g. five) could be used to retrieve a list of ranked 3D models, thus providing information about the overall shape of the complex in the query. Similar strategies were previously applied successfully in the case of SAXS, where an experimental one-dimensional SAXS profile can be searched against a library of simulated profiles to determine candidate 3D models [96,97]. Matching an "experimental" pattern to a library of known or simulated patterns, such as those generated from docked protein–protein complex models [98]or from conformations generated using NMA [75,99] continues to show its usefulness as an approach for identifying relevant shapes and conformations, without the need to process large amounts of data at great computational expense, for any type of experimental data.

Hybrid methods to shed light on disordered regions

Intrinsically disordered regions (IDRs) can constitute about 40% of eukaryotic proteome. They play a crucial role in signaling and regulation and are implicated in several diseases [100–102]. Despite their importance, the structural and functional understanding of disordered regions is limited as they pose immense challenges in experimental exploration. Being disordered, these proteins or regions show considerable flexibility, making them almost impossible to study using methods such as X-ray crystallography and cryo-EM. However, recent hybrid methods have enabled structural exploration of IDRs in several biological systems. For example, the C-terminal domain of RNA polymerase comprises a long, disordered tail that is involved in myriad of functions by interacting with several different proteins referred to as CTDSome. Jasnovidova et al. [103] used hybrid methodology, which combined X-ray crystallography, solution NMR, SAXS, and computational modeling to explore the structure and dynamics of CTDsome [103].

In addition, in a recent study, an ensemble of structures was determined for the disordered region of N-terminal activation domain (TAD) of the estrogen receptor [104]. Here, Peng et al. used all-atom explicit solvent MD simulations to generate a huge number of conformations for TAD. These conformations were then used in combination with SAXS data and hydroxyl radical protein footprinting data to generate a viable conformational ensemble. The obtained conformational ensemble was further validated by mutational experiments and ^{19}F NMR spectroscopy studies [104].

In another computational study on disordered domain of c-Src kinase, Shreshtha et al. [105] used Hamiltonian replica exchange molecular dynamics simulations to derive an ensemble of conformations that faithfully recapitulated the experimental observations obtained using SAXS, SANS, and NMR experiments [105]. This further shows the efficacy of hybrid methods combining advanced molecular simulations and experimental methods aimed at exploring solution dynamics, to obtain conformational information about intrinsically disordered proteins.

Hybrid methods to study dynamics

While many experimental techniques have been used to determine 3D structures, there are few that provide data characterizing the dynamics of biomolecules directly [8]. Among those, NMR is the most commonly used method that provides an atomic resolution insight into the solution state dynamics of macromolecules. NMR-based methods such as Nuclear Spin Relaxation, Residue Dipolar Coupling, and Exchange Spectroscopy provide information regarding biomolecular dynamics within a broad range of timescale from picoseconds to milliseconds [106]. However, the size of biomolecules that can be studied via NMR is usually limited to less than 30 kDa [107]. As a result, hybrid methods combining, X-ray crystallography, NMR, scattering studies, smFRET, high-speed Atomic Force Microscopy (HS-AFM), and MD simulations are increasingly being used to explore the dynamics of biological macromolecules, as the data from these sources may contain dynamic information at varying magnitudes and timescales [81,107–109].

In particular, application of AFM to study dynamics of large protein complexes has seen tremendous advancements with the development of HS-AFM [110]. HS-AFM enables extraction of not only topological but also dynamical features of the molecules embedded on the surface being scanned. The HS-AFM images are rich in features and provide a means to develop low-resolution structural model of biomolecules without the need of chemical stains or tags [111]. HS-AFM in combination with electron cryo-tomography (ECT) was used recently to understand the role of inner lumen proteins in the stability of microtubules in cilia and flagella [112].

Single-molecule FRET (smFRET) is another rapidly advancing technique developed to provide dynamical information about biological macromolecules [81,113]. Most experimental methods used for monitoring the dynamics of molecules in solution provide an average ensemble of conformations, and, thus, can give only limited information about the conformational heterogeneity of the molecules in solution [113]. SmFRET, on the other hand, provides dynamic information about a single molecule, which pairs well with computational modeling and MD simulations, to provide insight into the structure and dynamics of biomolecules. Recently Mazal et al. [114] explored domain motions in ClpB, a cellular machine that prevents aggregation of proteins within cells, using smFRET. They found fast timescale domain motions primarily between two conformations, which they computationally modeled using existing X-ray crystallography structures [114]. This led to the crucial mechanistic understanding of allosteric regulation in ClpB. Owing to its ability to track single molecules for long time duration and a comparatively inexpensive experimental setup, smFRET is emerging as a lucrative tool to study the dynamics of intrinsically disordered proteins [115]. Using a machine learning approach to combine FRET data with MD simulations and Markov state modeling, Matsunaga et al. explored the conformational transitions in the WW domain of the Formin Binding Protein [116].

Dynamics from EM images

As we have mentioned, given the nonstatic identity of biomolecular sample, the data that are derived from imaging experiments undoubtedly contains information about its conformational landscape and overall dynamics. To exploit this aspect, Jin et al. developed HEMNMA, where models that are generated from a reference atomic structure via their normal modes, can be iteratively selected through comparison of its projection images with the experimental single-particle cryo-EM 2D images [75]. Thus, an ensemble of models that describes the conformational landscape of the sample, as captured in the data, can be obtained [75,76]. The major advantage of this is that the conformational landscape can be described in a continuous manner, avoiding the limitations of assigning discrete numbers of classes during classification, which typically leads to the loss of information [117]. Another method that describes the conformational landscape continuously is manifold embedding. In the development of this method, Dashti et al. [118], extracted projection directions corresponding to continuous series of conformational fluctuations from ~850,000 cryo-EM snapshots of 80s ribosome from yeast [118,119]. Manifold embedding relies on the assumption that the data contain enough information to form a continuous trajectory or to characterize dynamics that are common to the ensemble.

Future of Hybrid Methods

Hybrid modeling methods have come a long way over the years and continue to provide crucial insights into the structure and dynamics of biomolecules. In this section, we will discuss the areas where hybrid methods could play a major role in near future.

X-ray free-electron laser single-particle imaging

Advances in single-particle imaging methods have provided new opportunities to characterize biomolecules and complexes, which are hard to crystallize. Previously, XFEL scattering experiments gained momentum as a viable technique when crystallized or partially crystallized samples were used [12,13,120]. XFELs allow for "diffraction-before-destruction," which could improve the quality of data by reducing radiation damage. However, with

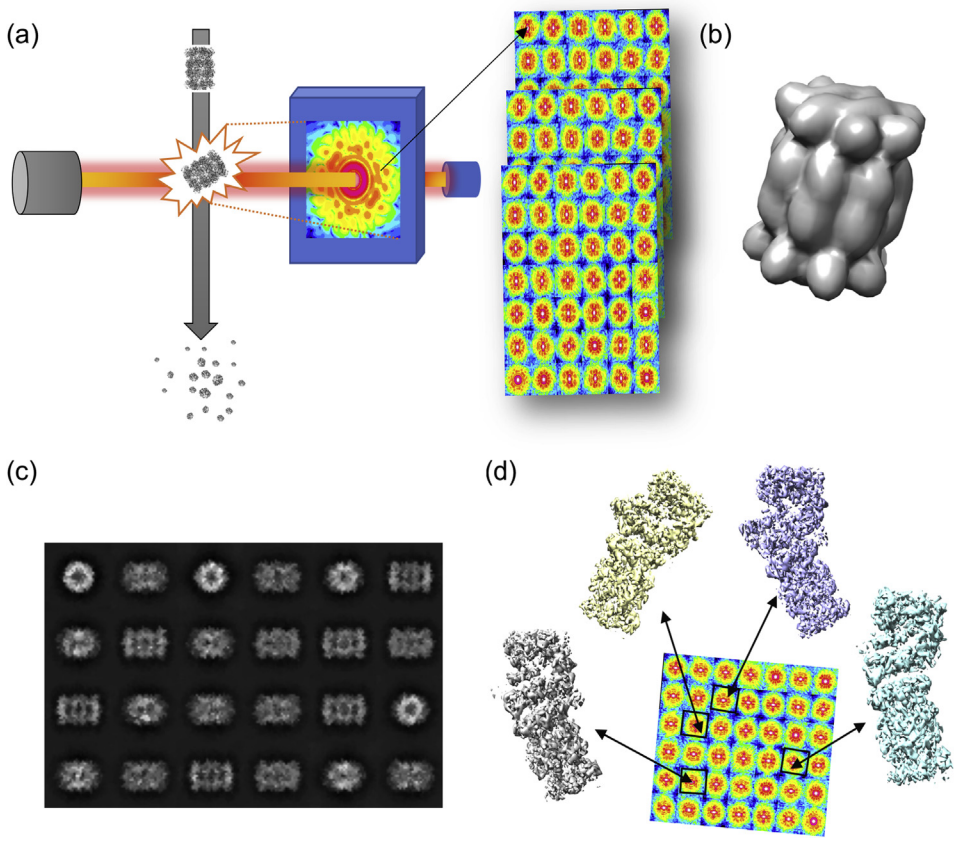


Fig. 2. Single-particle (SP) X-ray free electron laser (XFEL) scattering experiments produce data that can be used to probe biomolecules under non-crystalline conditions. (a) In the experiment, a very strong XFEL targets purified sample that is injected in a stream. Due to the short photon pulse (within 10 fs), observation is completed before the destruction of the sample. As the sample is not fixed, diffraction patterns of the sample can be obtained in Fourier space, corresponding to different orientations and conformations. (b) A sufficient number of patterns with adequate quality can be used to reconstruct a three-dimensional (3D) model through computational algorithms. (c) Two-dimensional diffractions could be converted to two-dimensional projection images in real space via phase retrieval for interpretation, assuming experimental conditions where the Ewald curvature can be ignored. (d) Hybrid methods involving molecular modeling techniques guided directly by the 2D experimental data can generate models which describe the conformational heterogeneity of the sample.

the single-particle imaging method, the orientation of the samples cannot be controlled, resulting in the lack of angle information that is particularly important for 3D structure reconstruction. XFEL data itself are in Fourier space, which means that it is affected by the phase problem that commonly affects most X-ray methods (Fig. 2a).

Single-particle XFEL-based 3D reconstruction methods can be thought of as extension of computational algorithms used in single-particle cryo-EM 3D reconstruction. First, the diffraction patterns are preprocessed through filtering and classification [121]. After a sufficient number of diffraction patterns has been classified, a 3D model can be reconstructed via algorithms such as projection matching; the expand, maximize, and compress algorithm; and other maximum likelihood approaches [122–125] (Fig. 2b).

Reconstructing 3D structures at a molecular resolution requires a large number of 2D experimental data, as well as higher beam fluence [126]. The current resolution range of 3D models obtained from XFEL single-particle studies is approximately ten to hundreds of nanometers; the giant mimivirus at 125 nm [123], coliphage PR772 at 9 nm [127,128] and 11.7 nm [129], Melbourne virus at 28 nm [130], and mammalian mitochondrion at 60 nm resolution [131]. The resolutions of these models are admittedly low, in comparison with other techniques, largely due to the XFEL beam being insufficiently strong. However, considering the early days of XFEL, and the three decades that it took for methods in single-particle cryo-EM to achieve atomic-level resolutions, both in terms of technology and analysis, further development of XFEL experimental techniques and computational analyses should be pursued.

Interpreting structure and dynamics directly from 2D XFEL data

The main aim of XFEL technology advancements is to be able to determine atomic-level 3D structures of samples of interest. However, combining computational molecular modeling, such as molecular dynamics, normal mode analysis and/or other types of structural data, with XFEL diffraction patterns is necessary to obtain structural information from the data that are currently available.

XFEL diffraction patterns can be used to retrieve meaningful 2D projection images and that can be used to interpret the dynamics of biomolecules under physiological conditions, assuming that the observed diffraction patterns are only at low angles, where the curvature of Ewald spheres can be ignored (Fig. 2c) [132]. Recently, Bränden et al. [133] performed the XFEL imaging of preformed bovine microtubules that were injected across an XFEL beam using a liquid microjet [133]. They sorted the captured diffraction patterns into class averages and merged these data to reconstruct a projection image of the microtubule. Essentially, they were able to produce an optimized diffraction pattern, which consisted of data merged from a total of 13,511 individual X-ray exposures. They performed the iterative phase retrieval by using a featureless tube as the initial model to retrieve 2D projection images in real space and validated their results using projection images generated from an oversimplified microtubule that they modeled, to interpret the structure of microtubules in noncrystalline conditions.

As a hybrid approach, matching experimental data to a set of known patterns is a useful strategy to identify relevant shapes and conformations in XFEL data, without the need for extensive computation (Fig. 2d). Tokuhisa et al. proposed a hybrid approach that involves generating a large number of models to identify the model structure that is in the best agreement with the experimental data [134]. Such a strategy avoids phase retrieval and the general problem of 3D reconstruction. The resulting protocol, given sufficient laser intensity, could annotate different conformations of biological molecules; the theoretical limit of distinguishing a conformational change in the diffraction patterns being within a few Å RMSD. The assumed fluence, $2-5 \times 10^{14}$ photons/μm^2, is much stronger than the currently attainable strength. For example, single-particle data were collected at the AMO end-station at Linac Coherent Light Source at ~10^{13} photons/μm^2 brilliance in 2017 [127]. However, the required condition could be reached in the near future with the development of new and improved instruments, such as a better beam focusing mirror.

Inspired by Tokuhisa et al. [134], Wang, and Liu proposed a hybrid approach whereby single-particle XFEL diffraction patterns can inform the best poses generated by the protein–protein docking program Zdock for several selected protein complexes [98]. In their method, the docking program generates several candidate models, and their single-particle diffraction patterns are simulated. These simulated patterns are then compared with a set of reference ("experimental") patterns, which in turn ranks the best matching complex orientation via a scoring function [98]. Furthermore, Nagai et al. explored the use of Gaussian mixture models (GMMs) [135] as a coarse-grained approach for XFEL analysis, which can quickly generate a large number of hypothetical structural models based on existing X-ray crystallography data, to interpret experimental data [136]. They addressed the need for speeding up exhaustive angular assignment when performing 3D reconstruction. GMMs were able to produce a large quantity of simulated data very quickly, with the potential for multiple conformations to be modeled. GMMs were also able to capture shape details at a reasonable resolution given that the current target systems in XFEL experiments are large macromolecular complexes, where atomic details are not essential.

In-situ/in-vivo structural biology

Visualization of biomolecules within cellular environment has always been one of the foremost areas of interests for biophysicists and has kept a special appeal over the years. This was one of the major reasons for the success and popularity of fluorescence probes such as green fluorescent protein. However, the resolution of fluorescence microscopy even with super-resolution techniques is limited to tens to hundreds of nanometers [137]. This is still far from the atomic resolution often desired for understanding the functional mechanisms of macromolecules. In this regard, there have been some recent exciting developments in the field of ECT, also referred to as cryogenic electron tomography (cryo-ET).

Conventionally, ECT has been used to visualize whole cells and subcellular organelles. However, with advancements in the direct electron detector and image processing algorithms, it is now possible to visualize macromolecular assemblies within cells in their native environment [138]. Although the resolution achieved for single biomolecular assemblies is still low (~1–10 nm), with advances in subtomogram averaging using multiple copies of same macromolecule in cell, the highest resolution attained by ECT has reached ~4 Å [139]. As part of the hybrid methods, ECT has already been proven crucial in determining the molecular architecture of nuclear pore complex and other macromolecular assemblies recalcitrant to any single biophysical method [63,140–142]. ECT has a distinct advantage that it can be used in cellular context and with further

advances in milling techniques such as focused ion beam milling [142] and novel innovations in experimental protocol such as cryo-lift [143], in conjunction with hybrid methods, determining near atomic resolution structure of biomolecular assemblies within cells seems on the horizon.

Perspective

Integrative/hybrid methods for understanding the structure and dynamics of biomolecules entail combining inputs from multiple experimental approaches computationally. The use of hybrid methods to understand increasingly complex biological systems has ushered an era of integrative structural biology. With the development of new methods such as XFEL single-particle imaging and time-resolved approaches, and immense advancements in the existing biophysical techniques such as cryo-EM, ECT, and smFRET, there are new challenges in terms of data analysis, which require specialized computational methods. These challenges can only be overcome by strong collaboration between scientific groups from diverse areas, including both experimental and computational sciences. The wwPDB Integrative/Hybrid task force workshop was a crucial step towards this direction [19]. Furthermore, standards need to be agreed upon for the representation and validation of hybrid models, along the lines of those within the Protein Data Bank, to allow for broader dissemination and utilization of the data generated from integrative protocols. In this regard, although a prototype is already in place [18], deliberate efforts are needed by the hybrid modeling community to actively engage in utilizing this prototype. This needs additional work, in the form of multidisciplinary training for researchers entering the field of integrative/hybrid structural biology from diverse backgrounds. Integrative/hybrid modeling methods hold great promise in the coming years and as with any new technique, they will only improve with time.

Acknowledgments

This work was supported by JSPS KAKENHI Grant Number 17K07305, 16K07286, 19H05781 and FOCUS for Establishing Supercomputing Center of Excellence. SPT is supported by the RIKEN Special Postdoctoral Fellowship.

Declaration of Interest

None.

Received 31 October 2019;
Received in revised form 20 January 2020;
Accepted 24 January 2020
Available online 13 February 2020

Keywords:
cryo-electron microscopy;
molecular dynamics;
flexible fitting;
normal mode analysis;
X-ray free-electron laser

Abbreviations used:
Cryo-EM, cryo-electron microscopy; Micro-ED, micro-electron diffraction; XL-MS, cross-linking mass spectrometry; SAXS, small-angle X-ray scattering; SANS, small-angle neutron scattering; smFRET, single-molecule förster resonance energy transfer; MC, Monte-Carlo; IDR, intrinsically disordered region; ECT, electron cryo-tomography; HS-AFM, high-speed atomic force microscopy; XFEL, X-ray free-electron laser; RMSD, root mean square deviation; GMM, Gaussian mixture model.

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