



## TEACHING TOOLS IN PLANT BIOLOGY™: LECTURE NOTES

# Computational Microscopy: Revealing Molecular Mechanisms in Plants Using Molecular Dynamics Simulations

### WHAT ARE COMPUTATIONAL MICROSCOPY AND MOLECULAR DYNAMICS? AND WHY USE THEM?

Virtually all processes in living organisms, from nutrient transport to the regulation of growth, are mediated by proteins. Gaining a detailed view of the biological processes occurring in plants requires understanding of the structure and function of the proteins involved in these processes. Sequence information is widely available for proteins across organisms, but structural information is still lacking, especially for plant proteins. Out of ~52 million protein sequences that are available in the UniProt database as of 2018, only 100,000 (0.2%) have structures deposited in the Protein Data Bank (PDB), and only ~4000 (4%) of these structures are of plant proteins. Structural biology has provided valuable insights and high-resolution views of the biophysical processes in plants, such as photosynthesis, hormone signaling, nutrient transport, and toxin efflux. However, structural biology only provides a few “snapshots” of protein structure, whereas *in vivo*, protein function involves complex dynamical processes such as ligand binding and conformational changes that structures alone are unable to capture in full detail.

Here, we present all-atom molecular dynamics (MD) simulations as a “computational microscope” that can be used to capture detailed structural and dynamical information about the molecular machinery in plants and gain high-resolution insights into plant growth and function. In addition to the background information provided here, we have prepared a set of tutorials that allow students to run and explore MD simulations of plant proteins.

### THE NEED FOR MD SIMULATIONS IN PLANT BIOLOGY

As computational tools become increasingly used across all technical fields, the need grows for scientists across disciplines, including plant biology, to be familiar with computing. This growth provides ample opportunities for work on the interface of biology and physics. Developing fundamental understanding of the underlying molecular mechanisms behind plant growth and physiological function requires a combination of biology and physics at the molecular scale. Effective integration of molecular modeling will allow scientists to bridge the gap between observable phenomena and the underlying molecular mechanisms that govern them. This has the potential to solve many large-scale problems, particularly involving sustainability and food security, via interventions at the molecular level.

Plant biology is indispensable in solving many grand challenges of the future, such as food security in a changing climate, improving agricultural productivity, and design of novel pesticides

to control weeds that are becoming resistant to currently existing pesticides. Fundamentally, each of these problems can be connected to the dynamics of biophysical processes in plants. MD simulations are a critical tool in understanding the atomic-level details of each of these problems. Genetic engineering has emerged as a potential method of improving crop yield and nutrient use efficiency. As gene-editing technology develops, the prospect of directly manipulating crop genomes to produce desired phenotypic effects such as improved photosynthetic efficiency, enhanced nutrient transport, and stress tolerance becomes increasingly realistic. However, without molecular-level details of biophysical processes in plants, it is difficult to predict *a priori* the specific mutations that will lead to a desired effect. Here, MD simulations can help fill in this gap in knowledge by identifying key protein residues that modulate various processes. In addition to genetic engineering, MD simulations can be used to aid small-molecule design for agricultural applications. Computational methods are already widely used in the pharmaceutical development pipeline, and similar approaches can be applied to agrochemicals as well. A fundamental understanding of plant growth and physiological function is undoubtedly important to address many critical problems of the future, and integration of molecular modeling can greatly enhance this understanding and aid in addressing grand challenges of the future.

### BASICS OF PROTEIN STRUCTURE

Proteins are long chains of amino acids found in all living organisms that adopt a three-dimensional structure in order to perform various functions. In computational microscopy, MD analyzes protein structures starting at the atomic level and integrates our understanding of the biophysical properties of their primary structure (amino acids) to an understanding of higher-order structures. There are four levels of protein structure. (1) Primary structure is the sequence of amino acids that make up the chain. There are 20 naturally occurring amino acids. (2) Secondary structure is composed of local structural elements. The two main secondary structure motifs found in proteins are  $\alpha$ -helices, which are right-handed corkscrew-like structures, and  $\beta$ -strands, which are regions of the amino acid chain with a linear structure. (3) Tertiary structure refers to the three-dimensional fold of a protein. For single-subunit (single-chain) proteins, this three-dimensional fold governs function. (4) Quaternary structure is the joining of several individual protein chains to make larger multiple-subunit proteins.

## THE INNER WORKINGS OF A COMPUTATIONAL MICROSCOPE

MD was first developed in the 1950s, when it was initially used to simulate the motion of hard spheres. The first MD study on a protein was published in 1977, in which the authors found that the structure of bovine pancreatic trypsin inhibitor undergoes local fluctuations about its experimentally determined structure. Since this landmark study, advances in simulation methodology as well as computer architecture have made MD simulations a powerful tool to investigate the dynamics of biomolecular systems.

### Initial Protein Coordinates

All-atom MD simulations require an initial set of coordinates (i.e., structure), topology information (i.e., atom connectivity), a force field describing interactions between atoms, and an integrator to update velocities and positions of each atom over time. For proteins, the initial coordinates can be obtained from an experimentally determined structure using x-ray crystallography, cryoelectron microscopy, or homology modeling, where the three-dimensional structure of a protein is determined using the known structure of a homologous protein (a protein sharing a common ancestor) as a template. More recently, evolutionary coupling-based methods have been used to predict the three-dimensional structure of a protein from sequence information as well as to enhance the sampling efficiency of MD simulations. Sequence-based structure prediction methods are particularly useful when structural information is limited but sequences are readily available. Topology input for simulations is generated from known covalent bonds in residues. In addition to the coordinate information, coordinate files (e.g., PDB files) will often include residue assignments that map each atom to its residue. Biomolecular simulation packages, such as AMBER, NAMD, Desmond, OpenMM, and GROMACS, are able to determine the topology of the protein based on this information.

### Force Fields

At the core of the simulation itself is the force field, which is used to calculate forces between each of the atoms in the simulation. Several force fields have been extensively tested and validated for biological systems. The interactions that are typically included in a force field are as follows.

#### Covalent Bonds

The energy of a covalent bond between any two atoms can be interpreted as the energy required to stretch or compress a “spring” between two atoms. This term is approximated as a harmonic potential:  $V_B = k(d_{ij} - d_0)^2$ , where  $k$  is a spring constant,  $d_{ij}$  is the distance between two atoms, and  $d_0$  is the optimal bond distance.

#### Bond Angles

The bond angle term describes the energy of a bending motion between any three atoms connected via covalent bonds. This can

be interpreted as the energy required to open or close a “spring hinge” with one atom at the vertex of the hinge and one atom on each end. Bond angle energy is represented as:  $V_A = k(\theta_{ijk} - \theta_0)$ , where  $k$  is again a spring constant,  $\theta_{ijk}$  is the angle formed by three atoms, and  $\theta_0$  is the optimal bond angle.

#### Dihedral Angles

The dihedral angle term describes the energy of a twisting motion between groups bonded to a pair of covalently bonded atoms. To visualize a dihedral angle twisting motion, imagine removing a long screw from a surface with a wrench. A dihedral angle would be the angle between a point on the surface some distance from the screw and the end of the wrench not in contact with the screw. The dihedral angle energy term describes the energy required to twist the wrench to a given angle. This term is represented as:  $V_D = v[1 + \cos(n\phi_{ijkl} - \phi_0)]$ , where  $v$  is a constant,  $\phi_{ijkl}$  is the dihedral angle between two planes formed by four bonded atoms, and  $\phi_0$  is the optimal dihedral angle.

#### Electrostatic Interactions

The electrostatics term describes electrostatic interactions between atoms that are not covalently bonded to each other. Each atom will have a charge due to bond polarity, but electrostatic interactions are most prominent between ions, such as sodium and chloride ions in a solution, or acidic and basic amino acids.

Electrostatics are described using Coulomb’s law:  $V_E = \frac{q_i q_j}{4\pi\epsilon_0 d_{ij}}$ , where  $q_i$  and  $q_j$  are the atomic charges and  $d_{ij}$  is the interatomic distance.

#### Van der Waals Interactions

Van der Waals interactions between nonbonded atoms are approximated as a Lennard-Jones potential. Examples of Van der Waals interactions are dispersive interactions between nonpolar molecules and induced-dipole interactions between polar and nonpolar molecules. The Lennard-Jones potential is:

$V_{LJ} = \sigma \left[ \left( \frac{A}{d_{ij}} \right)^{12} - \left( \frac{B}{d_{ij}} \right)^6 \right]$ , where  $\sigma$  is a parameter that roughly corresponds to interaction strength and  $A$  and  $B$  are excluded volume parameters that describe how close two atoms can approach each other before they repel.

The total potential energy is calculated by summing all the components:

$$V_{total} = \sum_{bonds} V_B + \sum_{angles} V_A + \sum_{dihedrals} V_D + \sum_{nonbonded} (V_E + V_{LJ})$$

From classical physics, the net force on any atom in the system is calculated as the gradient of potential energy with respect to the atomic positions:  $\vec{F} = -\nabla V$ . Using Newton’s second law of motion, the acceleration of each atom can be computed:  $\vec{F} = m\vec{a}$ . Using numerical integration techniques, the velocities and positions

of each atom can be computed as a function of time. To ensure numerical stability, the length of a time step in a typical MD simulation is  $\sim 2$  fs, so that each time step is shorter than the time scale of the fastest chemical bond vibration.

### Running MD Simulations

While various software packages exist for performing MD simulations, the protocols for preparing and running MD simulations follow the same basic steps of generating a topology file from an initial structure, adding “patches” or modifications to the protein such as terminal caps or disulfide bonds, and adding a solvent to mimic a physiological environment. The output of an MD simulation is a trajectory file that contains the position of each atom in the simulation at each time step. These data can be analyzed and visualized using software packages such as MDTraj and VMD. Additional details on the simulation and analysis protocols are provided in our NAMD and VMD tutorials.

### Limitations of MD Simulations

Like many experimental techniques, MD simulations are prone to statistical error and systematic error. Statistical error results from time-scale limitations. A single time step in an MD simulation is in the femtosecond range, whereas biophysical processes occur on microsecond to millisecond time scales. As a result, simulating biologically relevant processes is very difficult and requires large amounts of computing time. For an  $\sim 300$ -residue protein, a 500-ns simulation can take  $\sim 100$  h on a computer with modern graphical processing units. However, MD programs are highly parallelized (they can be run on multiple computers at once) and run on large supercomputing clusters such as Blue Waters. Running simulations across multiple computers and using advanced simulation methods speed up calculations by several orders of magnitude and allow us to observe microsecond time-scale biological processes in a short period of time.

Systematic error in MD simulations results from the limited accuracy of force fields used to compute potential energies between atoms. While force field accuracy has improved over time, they are approximations of the real system and thus have limitations. For instance, MD simulations assume that covalent bonds do not rearrange. This assumption is reasonable for simulating protein motions that do not involve chemical reactions, but it breaks down for enzymatic or photochemical processes. These require more complicated quantum mechanics/molecular mechanics methods.

### KEY ACHIEVEMENTS OF MD SIMULATIONS

MD simulations have been applied to solve various problems involving biophysical processes, such as protein folding, ligand binding, and conformational changes. These are all processes that are crucial for understanding biological function; however, experimental methods are unable to resolve dynamical information about these processes with high spatial and temporal resolution. Dynamical information not only enhances the fundamental understanding of biological processes but is also a valuable aid in designing regulatory mechanisms for biological

processes. MD studies have proven to be highly useful in gaining fundamental, atomic-level insights into various biophysical processes.

### Protein Folding

A long-standing question in biochemistry has been the process by which proteins fold into their native structures. Experimental methods are able to measure folding times of proteins, but without a molecular-level resolution of the process, it is impossible to know how folding occurs (i.e., what structures form first and how the protein adopts its final three-dimensional shape). Lindorff-Larsen et al. performed long time-scale simulations to study protein-folding processes of 12 different small proteins with varied structures. Although the native structures of all 12 proteins were known, details of the folding pathway were largely inaccessible using experimental methods. The authors ran multiple-millisecond-long MD simulations and observed multiple instances of each protein unfolding and folding. In addition to identifying key intermediates along the folding pathways, the authors were able to compute the folding times of each protein, which were validated against experimentally observed folding times. This study demonstrated the ability of MD simulations to make useful predictions about the long time-scale dynamics of small proteins.

### Ligand Binding

Ligand binding is a vital part of many biological processes in plants, such as hormone signaling, and a mode of action for many small-molecule inhibitors (i.e., drugs and agrochemicals). While various experimental methods are able to characterize the bound poses and affinities of protein-ligand interactions, they lack the ability to resolve kinetic information about the binding process. Numerous studies have employed MD simulations to characterize protein-ligand binding, primarily in the context of drug-target interactions. For instance, Shan et al. used MD simulations to characterize the binding process of the cancer drug dasatinib to its protein target. Using multiple MD simulations totaling  $\sim 115$   $\mu$ s of sampling, the authors were able to capture the binding pathway, reveal structural changes in the protein during the binding process, and investigate the role of water molecules during drug binding. Later studies have taken this idea further by performing extensive sampling of ligand binding processes to compute on and off rates of protein-ligand interactions. These MD studies of ligand binding are useful for predicting binding kinetics of small molecules, such as drugs and agrochemicals.

### Conformational Changes

The structure-function relationship of a protein is a central theme in structural and molecular biology. Critically, the functions of biomolecules are dependent not only on their native structures but on their ability to undergo conformational changes through an ensemble of structures. For instance, membrane transporters are known to transport small molecules through cell membranes via an “alternating access mechanism” in which the transporter cycles through inward-facing, occluded, and outward-facing states. A recent study by Selvam et al. revealed the complete

transport cycle of the bacterial peptide transporter  $\text{PepT}_{\text{So}}$ , which is a homolog of the nitrate transporter NRT1.1 in plants. A single crystal structure of this protein in inward-facing state is available, and previous experimental studies had used spectroscopy measurements to probe the kinetics of the conformational transitions. However, these experiments provide only the changes in specific residue distances over time. Using extensive MD simulations, the authors were able to resolve all the states of the transporter and short-lived intermediate structures along the transition paths between states that would ordinarily be inaccessible due to the difficulty of experimentally isolating short-lived states. Additionally, the authors employed a method called transition path theory to compute the time scales of transitions between the major states of the transporter. The same approaches can be used to characterize conformational dynamics of other proteins, such as hormone receptors, enzymes, and transcription factors, as well.

### RECENT APPLICATIONS OF MD SIMULATIONS IN PLANTS

Thus far, there have been few applications of MD simulations in plant biology, which can be partially attributed to the relatively small number of structures available for plant proteins. As previously mentioned, only ~4000 structures of plant proteins are available in the PDB, compared with ~40,000 structures of human proteins. Nonetheless, several recent MD studies on plant proteins and their homologs have shown MD to be an extremely valuable method to understand hormone signaling processes and nutrient transport in plants.

#### Brassinosteroid Signaling

Recent work by Moffett et al. used MD simulations to investigate mechanistic details of brassinosteroid signaling. Brassinosteroid signaling is known to be responsible for the regulation of plant growth and stress responses. It begins with the steroid hormone brassinolide binding to the extracellular domain of the receptor-like kinase BRASSINOSTEROID INSENSITIVE1 (BRI1), after which BRI1 associates with another kinase BRI1-ASSOCIATED KINASE1 (BAK1). Structures of both BAK1 and BRI1 are available, and the activation mechanism of human kinases has been studied extensively, but the exact mechanisms of plant kinase activation are unknown. MD simulations revealed that, in addition to phosphorylation, activation of BRI1 kinase requires breaking of a Lys-Glu charge-charge interaction that allows for unfolding of the  $\alpha$ -C helix of the kinase. These results are consistent with previous MD studies on human kinases. For the first time, molecular modeling was used to uncover high-resolution details about a crucial signaling pathway in plants.

In another study, Moffett et al. investigated the effects of S-glutathionylation on the conformational dynamics of BAK1. S-Glutathionylation is a post-translational modification in which a Cys residue on a protein is modified with a Glu-Cys-Gly peptide via a disulfide bond between the Cys residues. The authors performed MD simulations of BAK1 S-glutathionylated at three different positions (C353, C374, and C408). They found that glutathionylation at the C353 and C374 positions had little effect on the conformational dynamics of BAK1, but glutathionylation at the C408 position

stabilized the inactive state of BAK1, thus increasing the barrier for transition to the active state and decreasing the catalytic activity of the kinase. Additionally, they determined that this stabilization of the inactive state likely occurs via interactions between glutathione and the  $\alpha$ -C helix. Previous experimental work had implicated S-glutathionylation as a mode of allosteric regulation of BAK1, but for the first time, molecular modeling was able to gain atomic-level resolution into the mechanism by which this post-translational modification may decrease BAK1 activity.

#### Abscisic Acid Signaling

A long-standing problem in crop sciences is the regulation of drought resistance in plants, which is mediated by the abscisic acid (ABA) signaling pathway. The process begins with ABA binding to one of a class of receptors known as pyrabactin-resistant1-like (PYL) proteins. Upon binding of ABA, the receptor undergoes a conformational change that allows it to associate with Ser/Thr protein phosphatase 2C (PP2C) and inactivate it. Inactivation of PP2C in turn triggers a signaling cascade to control stomatal closure.

Several questions remain unanswered from the viewpoint of structural biology. (1) How does ABA bind to its receptor? (2) How do PYL proteins undergo conformational changes upon ABA binding? (3) How do Tyr nitration and other posttranslational modifications alter ABA signaling? Shukla and Zhao et al. investigated these questions using extensive MD simulations of the ABA binding process to PYL5.

This study identified that hydrogen bonding between ABA and a Lys residue in the binding pocket and water-mediated interactions with other binding pocket residues are key to ABA binding. Upon ABA binding, the “gate loop” of the receptor adopts a closed conformation. This study also found that a significant barrier to ABA binding is the exclusion of water from the binding pocket. The ABA binding pocket on PYL proteins is filled with water when not ABA-bound, and the binding process requires displacing these water molecules to allow ABA to form key interactions with binding pocket residues.

Furthermore, the authors investigated the effects of Tyr nitration on ABA binding. Previous PP2C inhibition assays determined that Tyr nitration inhibited ABA receptor activity, but the molecular origin was unclear. They also investigated the molecular basis for this inhibition by modifying the Tyr residues of ABA receptor to nitrotyrosine. MD simulations of nitrated PYL5 revealed that this modification prevented ABA from binding to PYL5, in turn rendering the gate loop unable to close. This detailed view of ABA perception by plants can aid the design of agrochemicals to inoculate crops to drought. (Note: In one of the accompanying Tutorials, students have the opportunity to explore the MD simulation of ABA binding to PYL5.)

#### SWEET Transporters

In addition to mediating cell signaling pathways, a critical role of proteins in plants is transporting nutrients across cell membranes. One family of plant proteins that performs this task is Sugars Will Eventually be Exported Transporters (SWEETs). These proteins play an important role in various physiological functions such as

plant growth, nectar production, and seed and pollen development. The structures of the SWEET transporters OsSWEET2b and AtSWEET13 have been solved in the inward-facing state, but the molecular mechanism of glucose recognition, binding, and transport by SWEETs remains unclear. Selvam et al. used MD simulations to capture the entire transport cycle of OsSWEET2b transporting glucose and were able to capture the transitions between the inward-facing, occluded, and outward-facing states, indicating that SWEETs transport glucose through an “alternating access” transport mechanism. In addition to identifying the conformational changes, the simulations were able to identify the specific changes in hydrogen bonding networks that mediate the transitions between the three major conformational states. This study also found that a set of hydrophobic residues inside the transport channel act as a “hydrophobic gate” that can restrict glucose movement. As the need to produce food increases with growing population, detailed knowledge of sugar transport mechanisms in plants can aid in genetically engineering crops with enhanced sugar transport and ultimately improve crop productivity.

## INTEGRATION OF SIMULATIONS AND EXPERIMENTS

A vital component of extensive MD studies is integration with experiments. Results from MD simulations are much more powerful if they are validated with experimental data, such as biophysical measurements. These measurements can also be used to improve the efficiency of MD simulations and the accuracy of MD results. There are a variety of methods to achieve this goal, including using results from biophysical measurements to improve the efficiency of simulations, incorporating experimental data into kinetic models constructed from simulation data, and using experimental data to validate and improve physical models themselves.

In addition to the validation of MD results using experiments, MD simulations also can be used to guide experimental studies, such as mutagenesis. A typical flow of information in combined MD simulation and mutagenesis studies is that mutagenesis determines the effects of mutations on some observable quantity, such as ligand binding affinity or functional activity, and MD simulations are used to interpret the results by providing atomistic details. Information can also flow in the reverse direction, in which results from MD studies are used to identify the important residues for a certain protein function and thus provide a starting point for mutagenesis studies. For instance, an MD study by Li et al. investigated water transport through five different membrane transporters. The authors identified a “channel-like” state of the transporter allowing for water permeation as well as key gating residues along the transport channel that modulate water permeation. A later study by Zeuthen et al. tested the predictions from this MD study by mutating the predicted gating residues and measuring water permeability. These studies serve as representative examples of integration between MD simulations and mutagenesis studies, but this integration has proven useful for many other systems.

## CONCLUSION

Despite the broad utility of molecular modeling and increased use of computational tools within the plant biology community, there

has thus far been little application of molecular modeling within plant biology. Due to the limitations of current experimental methods, molecular modeling is an indispensable tool in studying the dynamics of biophysical processes with high spatial and temporal resolution. MD simulations have already been applied to provide valuable insights into protein folding, drug binding, and protein conformational changes. As more structural information on plant proteins becomes available, ample opportunities exist to integrate MD simulations into plant biology as well. Application of MD simulations to plant biology has the potential to be a revolutionary force to drastically improve our understanding of the fundamental molecular biology of plants and address global issues concerning sustainability and food security.

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## RECOMMENDED READING

(This is a representative list of sources to help the reader access a huge body of literature. We apologize in advance to those whose work is not included.)

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