Best Practices for Quantification of Uncertainty and Sampling Quality in Molecular Simulations: v1.0

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Abstract   The quantitative assessment of uncertainty and sampling quality is essential in molecular simulation. Many systems of interest are highly complex, at the edge of current computational capacity, and simulators must understand and communicate statistical uncertainties so that 'consumers' of the data understand the meaning and limitations of the simulation data. This article covers key analyses appropriate for trajectory data generated by straightforward simulation methods such as molecular dynamics and (single Markov chain) Monte Carlo, as well as providing guidance for analyzing some 'enhanced' sampling approaches. We do not discuss *systematic* errors arising from inaccuracy in the chosen model or force field.

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1 Introduction: Scope and definitions

1.1 Scope

This article is geared toward simulation studies attempting to quantify observables and derive reliable estimates of uncertainty (e.g., error bars) based on 'standard' canonical sampling methods (e.g., molecular dynamics and Monte Carlo) and associated 'enhanced' sampling methods. Some problems and systems may be better studied with cruder techniques and analyses, which will not be covered here. This article also will not cover issues of systematic error arising from inaccuracy in force field (underlying model) or even from the simulation setup. Rather, we will take raw trajectory data at face value, assuming it is a valid outcome given the underlying model. We emphatically will *not* assume that a trajectory has been sufficiently 'equilibrated' or 'converged'.

1.2 Key Definitions

In order to make the discussion that follows more precise, we define key terms used in subsequent sections. We caution that while many of these concepts are familiar, our terminology follows the *International Vocabulary of Metrology* (VIM)[20], a standard that sometimes differs from the conventional or common language of engineering statistics. For additional information about or clarification of the statistical meaning of terms in the VIM, we suggest that readers consult the *Guide to the expression of uncertainty in measurement* (GUM)[19].

For clarity, we highlight differences between conventional terms and the VIM usage employed throughout this article. Of note, readers should study the term "standard uncertainty" which is sometimes estimated by (in common parlance) the "standard error of the mean"; however, the VIM term for the latter is the "experimental standard deviation of the mean." In cases of lexical ambiguity, the reader should assume that we hold to the definition of terms as given in the VIM.

The glossary is presented in a logical, rather than alphabetical order, in that each definition requires only those preceding it. We strongly encourage reading through the full glossary because of its structure and potentially unfamiliar terminology. Importantly, we also recommend reading the discussion that immediately follows, since this (i) explains the rationale for adopting the chosen language, (ii) discusses the limited relationship between statistics and uncertainty quantification, and (iii) thereby clarifies our perspective on best-practices.

1.2.1 Glossary of Statistical Terms

- **Random quantity**: A quantity whose numerical value is inherently unknowable or unpredictable. Observations or measurements taken from a molecular simulation are treated as random quantities[1].
- **True value:** The value of a quantity that is consistent with its definition and is the objective of an idealized measurement or simulation. The adjective "true" is often dropped when reference to the definition is clear by context[19, 20].
- **Standard Uncertainty**: Uncertainty in a result (e.g. estimation of a true value) as expressed in terms of a standard deviation[2].

- **Expectation value**: If $P(x)$ is the probability density of a continuous random quantity x, then the expectation value is given by the formula

$$\langle x \rangle = \int dx\, P(x) x. \tag{1}$$

In the case that x adopts discrete values $x_1, x_2, ...$, we instead write

$$\langle x \rangle = \sum_i x_i P(x_i). \tag{2}$$

- **Variance**:[3] Taking $P(x)$ as defined previously, the variance of a random quantity is a measure of how much it can fluctuate, given by the formula

$$\sigma_x^2 = \int dx\, P(x) \left(x - \langle x \rangle \right)^2. \tag{3}$$

If x assume discrete values, the coresponding definition becomes

$$\sigma_x^2 = \sum_i P(x_i) \left(x_i - \langle x \rangle \right)^2. \tag{4}$$

Remark: The standard deviation is the square root of the variance.

- **Arithmetic mean**: An estimate of the (true) expectation value of a random quantity, given by the formula

$$\bar{q} = \frac{1}{n} \sum_{k=1}^{n} q_k \tag{5}$$

where q_j is an experimental or simulated realization of the random variable and n is the number of samples.

Remark: This quantity is often called the "sample mean." Note that a proper realization of a random variable (with no systematic bias) will yield values distributed according to P, so $\bar{q} \rightarrow \langle q \rangle$ as $n \rightarrow \infty$.

- **Experimental standard deviation**[4] : An estimate of the (true) standard deviation of a random variable, given by the formula[5]

$$s\left(q_k \right) = \sqrt{\frac{\sum_{j=1}^{n} \left(q_j - \bar{q} \right)^2}{n - 1}} \tag{6}$$

Remark: This quantity is often called the "sample standard deviation."

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[1]Most molecular simulations (even those using pseudo-random number generators) are deterministic in that the sequence of visited states is generated by a fixed algorithm. As such, the simulation output is never truly random. In practice, however, the chaotic nature of the simulation allows for application of the principles of statistics to the analysis of simulation observations. Thus, observations/measurements taken at points along the simulation may be treated as random quantities. See Ref. [?] for more discussion of this rather deep point.

[2]The definition of standard uncertainty does not specify how to calculate the standard deviation. This choice ultimately rests with the modeler and should be dictated by the details of the uncertainty relevant to the problem at hand.

[3]The true probability density $P(x)$ is inherently unknowable, given that we can only collect a finite amount of data about x. As such, we can only estimate the expectation value of x and its variance.

[4] The term "experimental" can refer to simulated data, since these are the results of numerical experiments.

[5]The factor of $n - 1$ (as opposed to n) appearing in the demonitor of Eq. 6 is needed to ensure that the estimate is *unbiased*, meaning that on average $s(q_k)^2$ is equal to the true variance. Physically, we can interpret the –1 as accounting for the fact that one degree-of-freedom (e.g. piece of data) is lost via the appearance of $\bar{q}$ in the definition of $s(q_k)$. Equivalently, it accounts for the fact that the arithmetic mean is linearly correlated with each q_k (cf. Linearly Uncorrelated observables).

- **Linearly Uncorrelated observables**: If quantities q_j and q_k have mean values $\langle q_j \rangle$ and $\langle q_k \rangle$, then q_j and q_k are linearly uncorrelated if

$$\langle (q_j - \langle q_j \rangle)(q_k - \langle q_k \rangle) \rangle = 0 \qquad (7)$$

  where $<\star>$ denotes the (true) expectation value.

  Remark: Linear uncorrelation and independence of random variables are concepts that are often conflated. The latter amounts to the statement that the joint density of two random variables x and y can be decomposed as $P(x, y) = P(x)P(y)$, which is stronger than linear uncorrelation. Empirically testing for independence, however, is not practical, nor is it necessary for any of the estimates discussed in this work.

- **Experimental standard deviation of the mean**: An estimate of the standard deviation of the distribution of the arithmetic mean, given by the formula

$$s(\bar{q}) = \frac{s(q_k)}{\sqrt{n}}, \qquad (8)$$

  where the realizations of q_k are assumed to be linearly uncorrelated.

  Remark: This quantity is often called the "standard error." In the section on correlation analyses, we generalize this formula.

- **Raw data**: The numbers that the computer program directly generates as it proceeds through a sequence of states. For example, a MC simulation generates a sequence of configurations, for which there are associated properties such as the instantaneous pressure, temperature, volume, etc.

- **Derived observables**: Quantities derived from 'nontrivial' analyses of raw data, e.g. properties that may not be computed for a single configuration such as free energies.

- **Correlation time**: In time-series data of a random quantity $q(t)$ (e.g. a physical property from a MC or MD trajectory), this is the time τ over which $q(t)$ and $q(t + \tau)$ remain (linearly) correlated[6].

- **Two-sided confidence interval**: A statistically derived pair of lower and upper bounds between which the (true) expectation value of a random quantity is likely to fall, as quantified by a *confidence level* (typically given as a percentage, e.g. 95 %). For Gaussian-distributed data, the confidence level, in conjunction with the sample

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[6]Generally speaking, MC and MD trajectories generate new configurations from preceding ones. Thus, the correlation time can be interpreted as the time over which the system retains memory of its previous states. Such correlations are often **stationary**, meaning that τ is independent of t. Roughly speaking, the total simulation time divided by the longest correlation time yields an order-of-magnitude estimate of the number of (linearly) *uncorrelated* samples generated by a simulation.

size, determines the *coverage factor*, which is multiplied by $s(\bar{q})$ to assign the bounds of the confidence interval.

1.2.2 Terminology and its relation to our broader perspective on uncertainty

As surveyed by Refs. [19, 20], the discussion that originally motivated many of theses definitions appears rather philosophical. However, there are practical issues at stake related to both the content of the definitions as well as the need to adopt their usage. We review such issues now.

At the heart of the matter is the observation that any uncertainty analysis, no matter how thorough, is inherently subjective. This can can be understood, for example, by noting that the arithmetic mean is itself actually a random quantity that only approximates the true expectation value.[7] Because its variation relative to the true value depends on the number of samples (notwithstanding a little bad luck), one could therefore argue that a better mean is always obtained by collecting more data. We cannot collect data indefinitely, however, so the *quality* of an estimate necessarily depends on a *choice* of when to stop. Ultimately, this discussion forces us to acknowledge that *the role of any uncertainty estimate is to facilitate decision-making,* and, as such, the thoroughness of any analysis should be tailored to the decision at hand.

Practically speaking, the definitions as put forth by the VIM attempt to reflect this perspective while also capturing ideas that the statistics community have long found useful. For example, the concept of an "experimental standard deviation of the mean" is nothing more than the "standard error of the mean." However, the adjective "experimental" explicitly acknowledges that the estimate is in fact obtained from observation (and not analytical results), while the use of "deviation" in place of "error" emphasizes that the latter is unknowable. Similar considerations apply to the term "experimental standard deviation," which is more commonly referred to as the "sample standard deviation."

It is important to note that subjectivity as identified in this discussion does not arise just from questions of sampling. In particular, methods such as parametric bootstrap and correlation analyses (discussed below) invoke modeling assumptions that can never be objectively tested. Moreover, experts may not even agree on how to compute a derived quantity, which leads to ambiguity in what we mean by a "true value."[26] That we should consider these issues carefully and assess their impacts on any prediction is reflected in the definition of the "standard uncertainty," which does not actually tell us how to compute uncertainties. *Rather it is the task of the modeler to consider the impacts of their assumptions and choices when formulating a final uncertainty estimate. To this end, the language*

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[7]Notably, the same observation applies to the experimental standard deviation and the corresponding experimental standard deviation of the mean.

we use plays a large role in how well these considerations are communicated.

As a final thought, we reiterate that the goal of an uncertainty analysis is not necessarily to perform the most thorough computations possible, but rather to communicate clearly and openly what has been assumed and done. We cannot predict every use-case for data that we generate, nor can we anticipate the decisions that will be made on the basis of our predictions. The importance of clearly communicating therefore rests on the fact that in doing so, we allow others to decide for themselves whether our analysis is sufficient or requires revisiting. To this end, consistent and precise use of language plays an important, if understated role.

2 Best Practices Checklist

The self-contained checklist is presented on the following page.

3 Pre-simulation sanity checks and planning tips

Sampling a molecular system that is complex enough to be "interesting" in modern science is likely to be extremely challenging. Therefore, a small amount of effort spent planning a study can pay off many, many times over: in the worst case, weeks or months of simulation time followed by additional weeks or months of analysis can lead to insignificant conclusions in a poorly planned study.

If you read this guide through *before* performing a simulation, you will have a much better sense of the criteria applicable to your data – and which indeed *should* be applied by knowledgeable reviewers of your work. Thus we strongly advise understanding the concepts presented here as well as in related reviews [15, 19].

In a generic sense, the overall goal is to be able to draw statistically significant conclusions regarding a particular phenomenon or question of interest. And "good statistics" follow from repeated observations of a phenomenon, which can only happen if the simulation length *exceeds* the pertinent timescales.

One general strategy that will allow you to at least understand your data in a self-consistent way is to perform several repeats of the same simulation protocol: as described below, repeats can be used to assess variance in *any* observable, within the time you have run your simulation. When performing simulation repeats, it is sometimes suggested to use different starting states which are as diverse as possible; then, differences among the runs can be an indicator of inadequate sampling of the equilibrium distribution. Alternatively, performing multiple runs from the same starting state will
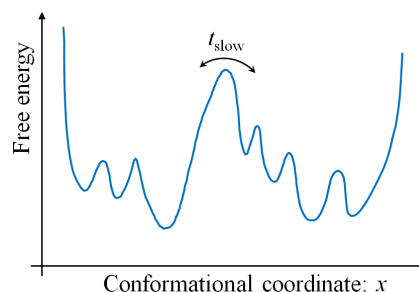

Figure 1. Schematic illustration of a free energy landscape dominated by a slow process. The timescales associated with a system will often reflect "activated" (energy-climbing) processes, although they could also indicate diffusion times for traversing a rough landscape with many small barriers. In the figure, the largest barrier is associated with the slowest timescale t_{slow}, and the danger for conventional MD simulations is that the total length of the simulation may be inadequate to generate the barrier crossing. The presence of stochasticity implies that even a simulation as long as t_{slow} may not yield the key event.

yield behavior particular to that starting state and potentially equilibrium if the runs are long enough.

What are the pertinent timescales? That's a question which has to be answered individually for each system. You will want to study the experimental and computational literature for your particular system, although we warn that a published prior simulation of a given length does not in itself validate a new simulation of a similar or slightly increased length. In the end, your data must be validated as well as possible by statistical analyses, such as those described below. Be warned that a system may possess states (regions of configuration space) that, although important, are *never* visited in a given simulation set because of insufficient computational time [15] – and this type of error will not be discovered through the analyses presented below. Finally, note that "system" here does not necessarily refer to a complete biological simulation (e.g. protein, solvent, ions, etc) - it can also refer to some subset of the simulation for which data is desired. For example, if one is only interested in the dynamics of a binding site in a protein, it probably is not necessary to observe the unfolding and refolding of that protein as well.

A toy model can generically illustrate timescales and their effects on sampling. Consider the double-well free energy landscape shown in Fig. 1, noting the slowest timescale is for crossing the largest barrier. Generally, you should expect that the value of *any* observable (e.g., *x* itself or another coordinate not shown or a function of those coordinates) will depend on which of the two dominant basins the system occupies; and, in turn, the equilibrium average of an observable will require sampling the two basins according to their equilibrium populations. In order to directly sample the equilibrium

QUANTIFYING UNCERTAINTY AND SAMPLING QUALITY IN MOLECULAR SIMULATION

☐ **Plan your study carefully: Start with pre-simulation sanity checks.** *Underlying concept:* There is no guarantee that any method, enhanced or otherwise, can sample the system of interest. See Sec. 3

  ☐ See best-practices papers on simulation background and planning/setup [https://github.com/MobleyLab/basic_simulation_training]

  ☐ Are system timescales known experimentally and feasible computationally based on published literature? If timescales are too long for straight-ahead MD, is there an enhanced method for which there are precedents for systems of similar complexity?

  ☐ Read up on sampling assessment – from this article or another source (e.g., [15]). Understanding uncertainty will help in the *planning* of a simulation.

  ☐ Consider multiple runs instead of a single simulation. Diverse starting structures enable a check on sampling for equilibrium ensembles, which should not depend on the starting structure. Multiple runs may be especially useful in assessing uncertainty for enhanced sampling methods.

  ☐ Check your code/method via a simple benchmark system. See: https://github.com/shirtsgroup/software-physical-validation

☐ **Perform simple, semi-quantitative checks which can rule out (but not ensure) sufficient sampling.** *Underlying concept:* It is easier to diagnose insufficient sampling than to demonstrate good sampling. See Sec. 4.

  ☐ Critically examine the time series of a number of observables – those of interest and then some more. Is each time series fluctuating about an average value or drifting overall? What states are expected and what are seen? Are there a significant number of transitions between states?

  ☐ If multiple runs have been performed, compare time series, distributions, and cluster populations from the different simulations.

  ☐ An individual trajectory can be divided into two parts and analyzed as if two simulations had been run.

  ☐ For individual runs, perform pairwise RMSD visual analysis as described below.

☐ **Remove an 'equilibration' (a.k.a. 'burn in', or transient) portion of a single MD or MC trajectory** and perform analyses only on the remaining 'production' portion of trajectory. *Underlying concept:* An initial configuration is unlikely to be representative of the desired ensemble and the resulting relaxation process must be accounted for. See Sec. 5.

☐ **Consider computing a quantitative measure of global sampling, particularly for a biomolecular system** - i.e., attempt to estimate the number of statistically independent samples in a trajectory. *Underlying concept:* Trajectory 'frames' (configurations) are highly correlated because one frame is generated from the preceding one, and estimating the degree of correlation is essential to understanding overall simulation quality. See Sec. 6.

☐ **Quantify uncertainty in specific observables of interest using a confidence interval.** *Underlying concept:* The statistical uncertainty in the estimate of an observable's *average* decreases as more independent samples are obtained, and can be much smaller than the standard deviation – which measures the range of variation in the observable. See Sec. 7.

☐ **Use special care and uncertainty analyses for enhanced sampling methods.** *Underlying concept:* The use of multiple, potentially correlated trajectories within a single enhanced-sampling simulation can invalidate the assumptions underpinning traditional analyses of uncertainty. See Sec. 8.

populations of the two basins, the length of a trajectory will have to be many times the slowest timescale, i.e. the largest barrier should be crossed multiple times. The relative populations of states are inferred from time spent in each state, which only will be representative if there are many transitions; put another way, the equilibrium populations follow from the transition rates [46] which can be estimated from multiple events. For completeness, we note that there is no guarantee that sampling of a given system will be limited by a dominant barrier. Instead, a system could exhibit a generally rough landscape with many pathways between states of interest. Nevertheless, the same cautions apply.

What should be done if a determination is made that a system's timescales are too long for direct simulation? The two main options would be to consider a more simplified ("coarse-grained") model or an enhanced sampling technique, bearing in mind that enhanced sampling methods are not foolproof but have their own limitations which should be considered carefully.

Lastly, whatever simulation protocol you pursue, be sure to use a well-validated piece of software [https://github.com/shirtsgroup/software-physical-validation]. If you are using your own code, check it against independent simulations on other software for a system that can be readily sampled.

4 Qualitative and semi-quantitative checks that can rule out good sampling

It is difficult to establish with certainty that good sampling has been achieved, but it is not difficult to *rule out* high-quality sampling. Here we elaborate on some relatively simple tests that can quickly bring out inadequacies in sampling.

Generally speaking, analysis routines that extract information from raw simulated data are often formulated on the basis of physical intuition about how that data should behave. Before proceeding to data analysis and uncertainty quantification, it is therefore useful to assess the extent to which data conforms to these expectations and the requirements imposed by either the modeler or the analysis routines. Such tasks help reduce subjectivity of predictions and offer insight into when a simulation protocol should be revisited to better understand their meaningfulness [26]. Unfortunately, general recipes for assessing data quality are impossible to formulate, owing to the range of physical quantities of interest to modelers. Nonetheless, several examples will help clarify the matter.

4.1 Zeroth-order system-wide tests

The simplest test for poor sampling is lack of equilibration: if the system is still noticeably relaxing from its starting conformation, statistical sampling has not even begun, and thus
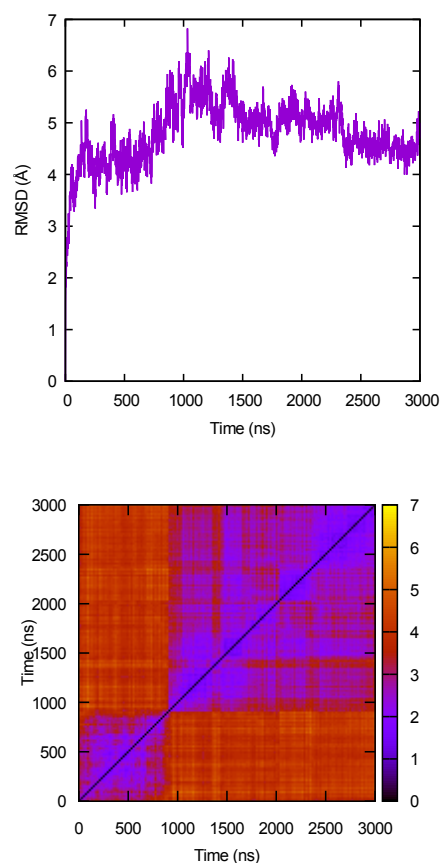

Figure 2. RMSD as a measure of convergence. The upper panel shows the α-carbon RMSD of the protein rhodopsin from its starting structure as a function of time. The lower panel shows the all-to-all RMSD map computed from the same trajectory. Data from Leioatts, et al [23].

by definition is poor. As a result, the very first test should be to verify that the basic equilibration has occurred. To check for this, one should inspect the time series for a number of simple scalar values, such as potential energy, system size (and area, if you are simulating a membrane or other system where one dimension is distinct from the others), temperature (if you are simulating in the NVE ensemble), and/or density (if simulating in the isothermal-isobaric ensemble). Often, simple visual inspection is sufficient to determine that the simulation is systematically changing, although more sophisticated methods have been proposed – see Sec. 5. If *any* value appears to be systematically changing, then the system is not equilibrated.

4.2 Tests based on configurational distance measures - e.g., RMSD for biomolecules

We will use the standard biomolecular RMSD (root mean-squared difference) as a generic distance measure for illustrative purposes. Alternatives to RMSD could be a dihedral-angle distance or another measure specific to your system of interest. Note that RMSD, like any distance in a high-dimensional space, becomes "degenerate" for larger values: given a reference configuration, there are a large number of configurations which differ from the reference by a given large RMSD. This is analogous to the increasing number of points in three-dimensional space with increasing radial distance from a reference point, except much worse because of the dimensionality. For a detailed exploration of expected RMSD distributions for biomolecular systems see the work of Pitera [28].

Some qualitative tools for assessing global sampling based on RMSD were reviewed in prior work [15]. The classic time series plot of RMSD with respect to a crystal or other single reference structure can immediately indicate whether the structure is still systematically changing. Although this kind of plot was historically used as a sampling test, it should really be considered as another equilibration test like those discussed above. Moreover, it's not even a particularly good test of equilibration, because the degeneracy of RMSD means you can't tell if the simulation is exploring new states that are equidistant from the chosen reference. The upper panel of Figure 2 shows a typical curve of this sort, taken from a simulation of the G protein-coupled receptor rhodopsin [23]; the curve increases rapidly over the few nanoseconds and then roughly plateaus. It is difficult to assign meaning to the other features on the curve.

A better RMSD-based convergence measure is the all-to-all RMSD plot; taking the RMSD of each snapshot in the trajectory with respect to all others allows you to use RMSD for what it does best, identifying very similar structures. The lower panel of Figure 2 shows an example of this kind of plot, applied to the same rhodopsin trajectory. By definition, all such plots have values of zero along the diagonal, and occupation of a given state shows up as a block of similar RMSD along the diagonal; in this case, there are 2 main states, with one transition occuring roughly 800 ns into the trajectory. Off diagonal "peaks" (regions of low RMSD between structures sampled far apart in time) indicate that the system is revisiting previously sampled states, a necessary condition for good statistics. In this case, the initial state is never sampled after the first transition, but there are a number of small transitions within the second state.

4.3 Testing for linear behavior in material properties

In many cases, data analysis relies on determining or extracting information from a regime in which data is expected to behave linearly. In the context of materials science, determining the yield-strain ϵ_y (loosely speaking, the deformation at which a material fails) provides one such example. In particular, intuition and experiments tells us that upon deforming a material by a fraction $1 + \epsilon$, it should recover its original dimensions if $\epsilon \leq \epsilon_y$ and have a residual strain $\epsilon_r = \epsilon - \epsilon_y$ if $\epsilon \geq \epsilon_y$ [27]. Thus, residual-strain data should exhibit bilinear behavior, with slopes indicating whether the material is in the pre- or post-yield regime.

In experimental data, these regimes are generally distinct and connected by a sharp transition. In simulated data, however, the transition in ϵ_r around yield is generally smooth and not piecewise linear, owing to the timescale limitations of MD. Thus, it is useful to perform analyses that can objectively identify the asymptotic regimes without need for input from a modeler. One way to achieve this is by fitting residual strain to a hyperbola. In doing so, the proximity of data to the asymptotes illustrates the extent to which simulated ϵ_r conforms to the expectation that $\epsilon_r = 0$ when $\epsilon < \epsilon_y$. See Fig. 3 and Refs. [26, 27] for more examples and discussion.

4.4 Assessing Convergence

When measuring any property it typically takes multiple measurements to get some idea of the uncertainty involved. For example, when trying to determine the probability that an unbiased coin will flip heads or tails, a single flip (measurement) will always yield an incorrect answer since it will either be 1.0 for heads or 1.0 for tails. As more flips (measurements) are made, the probability will eventually converge to the correct answer. Convergence in the context of biomolecular simulations typically refers to a measurement of a certain property converging to the "correct" value. However, since in many cases the "correct" value is not known *a priori*, convergence is
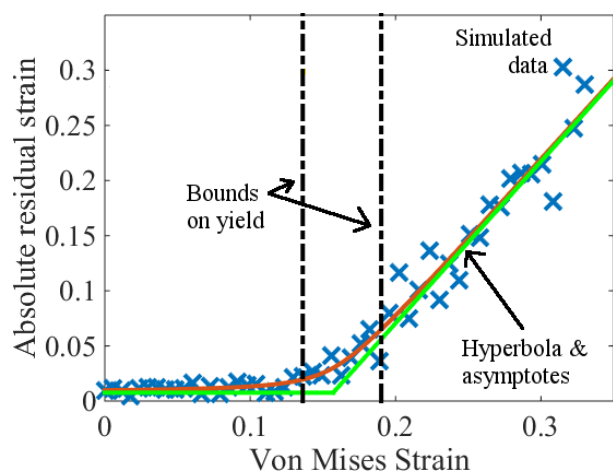
Figure 3. Yield strain tn ϵ_r as a function of applied strain ϵ. Blue × denote simulated data, whereas the smooth curve is a hyperbola fit to the data. The green lines are asymptotes; their intersection can be taken as an estimate of ϵ_y. Bounds on yield are computed by the synthetic data method discussed in the next section. *From, "Estimation and uncertainty quantification of yield via strain recovery simulations," P. Patrone, CAMX 2016 Conference Proceedings. Reprinted courtesy of the National Institute of Standards and Technology, U.S. Department of Commerce. Not copyrightable in the United States.*
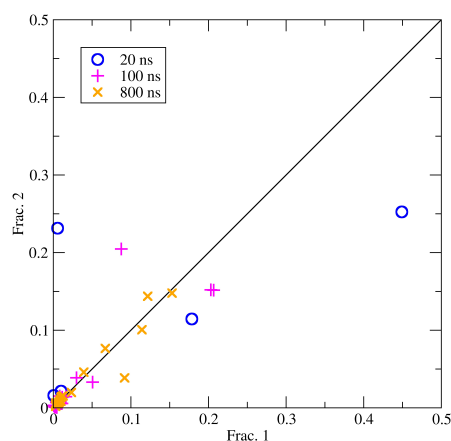

Figure 4. Combined clustering between two independent trajectories as a measure of convergence. The X axis is the population of a cluster from trajectory 1, while the Y axis is the population of that cluster from trajectory 2. Cluster populations are show after 20, 100, and 800 ns of sampling. The simulations used to generate the data used in this plot are described in Roe et al.[32]

also used to refer to the overlap of two or more independent measurements of the same property to a single value. If a measure is not converged it is a strong indication that sampling is poor. There are two common ways to try to obtain independent measurements. Arguably the best way is to have multiple independent simulations, each with varying initial starting conditions. Ideally these starting conditions should in some way span the space to be sampled; this way one can have confidence that simulations are not being trapped in a local minimum. For example, say the goal is to sample the phi and psi torsions of alanine dipeptide: the phi and psi angles for one simulation could be started from an alpha-helical conformation, while another simulation could be started from a polyproline II conformation. It is important to note that the starting conditions only need to be varied enough so that the desired space is sampled. For example, if the goal is to sample protein folding and unfolding, there should be some simulations started from the folded conformation and some from the unfolded, but if it is not important to consider protein folding no initial conformation needs to be unfolded.

Another way to obtain independent measurements is to divide a single simulation into two or more subsets. However this can at times be problematic because it can be more difficult to tell if the system is biased by initial conditions (e.g. trapped in a local energy minimum) since there is only a single starting point. Those employing this approach should take extra care to assess their results (see in particular sections on 'block averaging' below).

There are two simple ways to compare independent measurements of a property. The simplest is to compare the arithmetic means and standard deviations. If these values do not overlap then convergence has not been achieved. However, since this assumes that the values are normally distributed, a better way is compare the overlap of the probability distributions (i.e. the histograms). This can be done via Kullback-Leibler or Jensen-Shannon divergence.

4.4.1 Combined Clustering

One useful technique for evaluating convergence of structure populations is so-called "combined clustering". Briefly, in this method two or more independent trajectories are combined into a single trajectory (or a single trajectory is divided into two or more parts), on which cluster analysis is performed. The resulting clusters are then split according to the trajectory (or part of the trajectory) they originally came from. If simulations are converged then each part will have similar populations for any given cluster. Indications of poor convergence are large deviations in cluster populations, or clusters that show up in one part but not others. Figure 4 shows results from combined clustering of two independent trajectories as a plot of cluster population fraction from the first
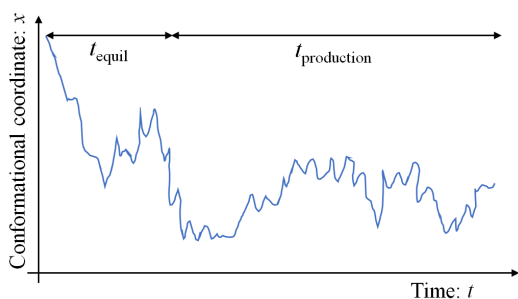
Figure 5. The equilibration and production segments of a trajectory. "Equilibration" over the time t_{equil} represents transient behavior while the initial configuration relaxes toward configurations more representative of the equilibrium ensemble. Readers are encouraged to select t_{equil} in a systematic way based on published literature. If you find strong sensitivity of "production" data to the choice of t_{equil}, this suggests additional sampling is required.

trajectory compared to the second. If the two independent trajectories are perfectly converged then all points should fall on the X=Y line. As simulation time increases the cluster populations from the independent trajectories are in better agreement, which indicates the simulations are converging. For another example of performing combined cluster analysis see Bergonzo et al.[2]

5 Determining and removing an equilibration or 'burn-in' portion of a trajectory

The 'equilibration' or 'burn-in' time t_{equil} represents the initial part of a single continuous trajectory (whether from MD or MC) that is *discarded* for purposes of data analysis of *equilibrium properties*; the remaining trajectory data is often called 'production' data. See Fig. 5. Discarding data may seem counter-productive, but there is no reason to expect that the initial configurations of a trajectory will be important in the ensemble ultimately obtained. Including early-time data, therefore, can systematically *bias* results.

Examples can help to illustrate the idea. In the biomolecular realm, consider the initial configuration of a protein, which has been energy-minimized and otherwise relaxed from a crystal structure. Such an initial structure might seem to be intrinsically valuable, but remember that configurations important in a crystal environment may not be equally pertinent to an aqueous environment due to e.g. crystal packing etc. Also, any energy minimization/relaxation that has been performed likely will be very local and keep the configuration within or nearby the initial energy basin. Moreover, the force field used to represent interactions will in general not be identical to that used to as part of the crystal structure solution. See the RMSD trace in Fig. 2, and note the very rapid RMSD increase in the first ~200 ns. This indicates that

the initial conformation was not favorable for the given conformation/simulation conditions. This rapid relaxation to a new conformation is inherently a non-equilibrium process and should not be included in the calculation of equilibrium properties. In materials science, if a simulation is designed to study thermally induced fluctuations or defects in a (mostly) ordered system, an initial fully ordered configuration is not likely to be representative of the targeted ensemble.

Accepting that some data should be discarded, it is not hard to see that we want to avoid discarding too much data, given that many systems of interest are extremely expensive to simulate. Putting those two competing goals into statistical language, we want to remove bias but also minimize uncertainty (variance).

Before briefly describing a procedure for estimating the optimal equilibration time, it should be emphasized that the very notion of separating a trajectory into equilibration and production segments only makes sense if the system has indeed reached configurations important in the equilibrium ensemble. While it is generally impossible to guarantee this has occurred, some easy checks for determining that this has not occurred are described in Sec. 4. *It is essential to perform those basic checks before analyzing data with a more sophisticated approach* that may assume a trajectory has a substantial amount of true equilibrium sampling.

The essential ideas of equilibration analysis are discussed carefully in prior work [7, 43]. The key procedure is to analyze data as a function of the amount of data removed - i.e., as t_{equil} increases from zero. As described by Chodera [7], both the bias and the variance can be monitored, as well as the effective sample size, which is roughly quantified by the total simulation time considered divided by the auto-correlation time. Chodera indicates that the effective sample size peaks at the optimal t_{equil}, making the latter easy to discern. We caution that estimating the correlation time may require care, and readers may want to consider the global 'decorrelation time' [24] described in Sec. 6. Further, if values of observables estimated from the production phase depend sensitively on the choice of t_{equil}, it is likely that further sampling is required.

6 Quantification of Global Sampling

With ideal trajectory data, one would hope to be able compute arbitrary observables with reasonably small error bars. During a simulation, it is not uncommon to monitor specific observables of interest, but after the data is obtained, it may prove necessary to compute observables not previously considered. These points motivate the goal of estimating global sampling quality, which can be framed most simply in the context of single-trajectory data: "Among the very large number

of simulation frames (snapshots), how many are statistically independent?" From a dynamical perspective, which also applies to Monte Carlo data, how long must one wait before the system completely loses memory of its prior configuration? The methods noted in this section build on ideas already presented in Sec. 4 on qualitative sampling analysis, but attempt to go a step further to quantify sampling quality.

We emphasize that *no single method described here has emerged as a clear best practice.* However, because the global assessment methods provide a powerful window into overall sampling quality – which could easily be masked in the analysis of single observables (Sec. 7) – we strongly encourage their use. The reader is encouraged to try one or more of the approaches in order to understand the limitations of their data.

6.1 Scope and a key caveat

The discussion here will focus largely on biomolecular systems, or more precisely, on systems for which it is straightforward to define a meaningful scalar distance between configurations.

A key caveat is needed before proceeding. Analysis of trajectory data generally cannot make inferences about parts of configuration space not visited [15]. It is generally impossible to know whether configurational states absent from a trajectory are appropriately absent because they are highly improbable (extremely high energy) or because the simulation simply failed to visit them because of a high barrier or bad luck.

6.2 Global sampling assessment for a single trajectory

Two methods applicable for a single trajectory have been previously introduced by some of the authors, exploiting the fact that trajectory correlations are sequential. That is, each configuration evolves from and is most similar to the immediately preceding configuration. This picture holds for standard MD and Markov-chain MC.

Lyman and Zuckerman proposed a global "decorrelation" analysis by mapping a trajectory to a discretization of configuration space and analyzing the resulting statistics [24]. Configuration space is discretized into bins based on Voronoi cells of structurally similar configurations - e.g., based on an RMSD criterion. The analysis method is based on the observation that the variance for any bin of a multinomial distribution is known, given a specified number of independent samples drawn from the discretized distribution. The knowledge of the expected variance allows testing of increasing waiting times between configurations drawn from the trajectory to determine when and if the variance approaches that expected

for independent samples. The minimum waiting time yielding agreement with ideal statistics yields an estimate for the decorrelation/memory time, which implies an overall effective sample size.

A second method, employing block covariance analysis (BCOM), was presented by Romo and Grossfield [35] building on ideas by Hess [17]. In essence, the method combines two standard error analysis techniques, block averaging [12] and bootstrapping [11], with a quantitative assessment of the similarity of modes determined from principal component analysis, covariance overlap [17]. The principal components are computed from subsets of the trajectory, and the similarity of the modes evaluated as a function of subset size; as the subsets get larger, the resulting modes get more similar. This is done both for contiguous blocks of trajectory data (block averaging), and again for randomly chosen subsets of trajectory frames (bootstrapping); taking the ratio of the two values as a function of block size yields the degree of correlation in the data. Fitting that ratio to a sum of exponentials allows one to extract the relaxation times in the sampling. The key value of this method over others is that it implicitly takes into account the number of substates; the longest correlation time is the time required not to make a transition, but to sample a scattering of the relevant states.

These methods are implemented as part of LOOS [34, 36].

6.3 Global sampling assessment for multiple independent trajectories

When sampling is performed using multiple independent trajectories (whether MD or MC), additional care is required. Analyses based solely on the assumption of sequential correlations may break down because of the unknown relationship between separate trajectories.

Zhang et al. extended the decorrelation/variance analysis noted above, while still retaining the basic strategy of inferring sample size based on variance [45]. To enable assessment of multiple trajectories, the new approach focused on conformational state populations, arguing that the states fundamentally underlie equilibrium observables. Employing a fairly simple kinetic-clustering technique to automatically define states, the approach then uses the variances in state populations among trajectories to estimate the effective sample size.

Nemec and Hoffmann proposed related sampling measures geared specifically for analyzing and comparing multiple trajectories [25]. These measures again do not require user input of specific observables but only a measure of the difference between conformations, which was taken to the be the RMSD. Nemec and Hoffmann provide formulas for quantifying the conformational overlap among trajectories (were

the same configurational states sampled?) and the density agreement (were conformational regions sampled with equal probabilties?).

6.4 Global sampling assessment for enhanced sampling methods

The family of enhanced equilibrium sampling methods, including replica exchange and variants [38–40], metadynamics [6, 22], adaptive biasing force [8–10] among other methods, are complex and the resulting data may have a highly nontrivial correlation structure. In replica exchange, for example, the ensemble at a temperature of interest will be based on multiple return visits of different sequentially correlated trajectories.

Given the subtleties of these sampling approaches, we suggest taking a 'bottom line' approach, and assessing sampling based on multiple independent runs. The variance among these runs, if the approach is not biased, should be a measure of the overall sampling. Hence any method applicable to multiple trajectories should be valid for analyzing multiple runs of an arbitrary method. A caveat for the approach of Zhang et al. [45] is that some dynamics trajectory segments would be required to perform state construction by kinetic clustering.

7 Computing error in specific observables

7.1 Basics

"What error bar should I report?" Here we address this simple but critical question.

In general, there is no one-best practice for choosing error bars. However, in the context of simulations, we can nonetheless identify common goals when reporting such estimates: (i) to help authors and readers better understand uncertainty in data; and (ii) to provide readers with realistic information about the reproducibility of a given result.

With this in mind, we recommend the following: (a) in fields where there is a definitive standard for reporting uncertainty, the authors should follow existing conventions; (b) otherwise, such as for biomolecular simulations, *authors should report (and graph) their best estimates of 95% confidence intervals.* As explained in the glossary above, a 95 % confidence interval is a range of values that is expected to bracket 95 % of the computed predictions *if statistically equivalent simulations are repeated a large number of times;* (c) when feasible and especially for a small number of independent measurements (n < 10), authors should consider plotting all of the points or a histogram instead of an average with error bars.

We emphasize that as opposed to standard uncertainties [$s(\bar{q})$], confidence intervals have several practical benefits that justify their usage. In particular, they directly quan-

tify the statistical frequency with which we expect a given outcome, which is more relatable to everyday experience than moments of a probability distribution. As such, confidence intervals can help authors and readers better understand the implications of an uncertainty analysis. Moreover, downstream users/readers of a given paper may include less statistically-oriented readers for whom confidence intervals are a more meaningful measure of variation.

In a related vein, error bars expressed in integer multiples of $s(\bar{q})$ can be misinterpreted as unrealistically under or overestimating uncertainty if taken at face value. For example, reporting $3s(\bar{q})$ uncertainties for a normal random variable amounts to a 99.7 % confidence interval, which is likely to be a significant overestimate for many applications. On the other hand, $1s(\bar{q})$ uncertainties only correspond to a 68 % confidence interval, which may be too low. Given that many readers may not take the time to make such conversions in their heads, we feel that it is safest for modelers to explicitly state the confidence level of their error bar or reported confidence interval.

In recommending 95 % confidence intervals, we are admittedly attempting to address a social issue that nevertheless has important implications for science as a whole. In particular, the authors of a study and the reputation of their field do not benefit in the long run by under-representing uncertainty, since this may lead to incorrect conclusions. But perhaps just as importantly, many of the same problems can arise if uncertainties are reported in a technically correct but obscure and difficult-to-interpret manner. For example, $1s(\bar{q})$ error bars may not overlap and thereby mask the inability to statistically distinguish two quantities, since the corresponding confidence intervals are only 68 %. With this in mind, we therefore wish to emphasize that visual impressions conveyed by figures in a paper are of primary importance. Regardless of what a research paper may explain carefully in text, error bars on graphs create a lasting impression and must be as informative and accurate as possible. If 95 % confidence intervals are reported, the expert reader can easily estimate the smaller standard uncertainty (especially if it is noted in the text), but showing a graph with overly small error bars is bound to mislead most readers – even experts who do not search out the fine print.

As a final note, we remind readers that only significant figures should be reported. Additional figures beyond the precision implicit in the uncertainty are unhelpful at best, and potentially misleading to readers who may not be aware of the limitations of simulations or statistical analyses generally.

7.2 Overview of procedures for computing a confidence interval

We remind readers that before attempting to quantify uncertainty via an error bar, the semi-quantitative checks on sampling (Sec. 4) should be performed. If the observable of interest is not fluctuating about a mean value but largely increasing or decreasing during the course of a simulation, it is unlikely that a reliable quantitative estimate for the observable (or an error bar) can be obtained.

For observables passing the qualitative tests noted above in Sec. 4, we advocate obtaining confidence intervals in one of two ways:

- For observables that are approximately Gaussian-distributed (or assumed to be, due to lack of information), an appropriately chosen *coverage factor k* (typically about 2, but see Sec. 7.6) multiplying the standard uncertainty s^* by k yields an estimate for a 95 % confidence interval.
- For non-Gaussian observables, a *bootstrapping* approach (described below) should be used. An example of a potentially non-Gaussian observable is a rate-constant, which must be positive but could exhibit significant variance, so a confidence interval estimated with a coverage factor may lead to an unphysical negative lower limit. Multimodal distributions certainly are not Gaussian. Bootstrapping does not assume an underlying distribution but instead constructs a confidence interval based on the recorded data values, and the limits cannot fall outside the extreme data values. Bootstrapping (or block averaging) is also necessary for cases for derived observables; this is true for quantities such as time correlation functions and anything derived from them.

Below we describe approaches for estimating the standard uncertainty $s(\bar{q})$ from a single trajectory with a coverage factor k as well as the bootstrapping approach for direct confidence-interval estimation. Whether using a coverage factor and standard uncertainty or bootstrapping, one requires an estimate for the independent number of observations in a given simulation. This requires care, but may be accomplished based on the effective sample size described in Sec. 6, via block averaging, or by analysis of a time-correlation function. However, these methods have their limitations and must be used with caution. Primarily, they require the user to look at the correct quantity in order to produce the correct answer. Both block averaging and autocorrelation analysis will produce different effective sample sizes for different quantities; to produce robust answers, one must identify and track the slowest relevant degree of freedom in the system, which can be a non-trivial task. Even apparently fast-varying properties may have significant statistical error if they are coupled to slower varying ones, and this error in uncertainty estimation may not be readily identifiable by solely examining the fast-varying time series.

In the absence of a reliable estimate for the number of independent observations, one can perform n independent simulations and calculate the standard deviation $s(q_k)$ for quantity q (which could be the ensemble average of a raw data output or a derived observable) among the n simulations, yielding a standard uncertainty of $s(\bar{q}) = s(q_k)/\sqrt{n}$. When computing the uncertainty with this approach, it is important to ensure that each starting configuration is also independent or else to recognize and report that the uncertainty refers to simulations started from a particular configuration. The means to obtain independent starting configurations is system-dependent, but might involve repeating the protocol used to construct a configuration (solvating a protein, inserting liquid molecules in a box, etc.), using a new random seed. However, readers are cautioned that *for complex systems, it may be effectively impossible to generate truly independent starting configurations pertinent to the ensemble of interest.* For example, a simulation of a protein in water will nearly always start from the experimental structure, which introduces some correlation in the resulting simulations even when the remaining simulation components (water, salt, etc) are regenerated *de novo*.

7.3 Block averaging method for estimating the standard uncertainty

The standard uncertainty of a simulation observable can be estimated from the experimental standard deviation of the observable divided by the square root of the number of independent samples. Due to correlation in the tape of simulation output from either MD or MC simulation, the number of independent samples in a simulation is neither equal to the number of observations nor known *a priori*. The block averaging technique can be used instead of the individual observations to account for correlation in samples, while using all data, subject only to the constraint that the "blocks" are large enough to be essentially uncorrelated[12–15].

Briefly, the set of N observations $\{q_1, ..., q_N\}$ are converted to a set of M "block averages" $\left\{q_1^b, ..., q_M^b\right\}$, where a block average q_k^b is the arithmetic mean of n (the block size) sequential measurements of q:

$$q_k^b = \frac{\sum_{j=1+(k-1)n}^{kn} q_k}{n} \qquad (9)$$

From this set of block averages, one may then compute the arithmetic mean of the block averages, $\bar{q}^b$, which is an estimator for $\langle q \rangle$. (We note that for $mod(N, n) = 0$, then $\bar{q}^b = \bar{q}$, as the entire set of q_k is used. This is *not* true if N is not evenly

divisible by either *M* or *n*.) Following, one computes the experimental standard deviation of the block averages, $s\left(\bar{q}^b\right)$ using Eq. 6. Lastly, the standard uncertainty of $\bar{q}^b$ is just the experimental standard deviation of the mean given the set of *M* block averages:

$$s\left(\bar{q}^b\right) = \frac{s\left(q_k^b\right)}{\sqrt{M}} \tag{10}$$

This standard uncertainty may then be used to calculate a confidence interval on $\bar{q}^b$.

It is crucial when performing this analysis to do so systematically as a function of block size; as the blocks get longer, the block averages should decorrelate and $s\left(\bar{q}^b\right)$ should plateau [12, 15]. Another approach is to measure the block correlation and to use it to improve the selection of the block size and, hence, uncertainty estimate [21]. We stress that this final step of adjusting the block size and recomputing the block standard uncertainty is absolutely necessary. Otherwise, the blocks may be correlated, yielding an uncertainty that is not meaningful.

7.4 Autocorrelation method for estimating the standard uncertainty

An alternative to the more common block averaging technique is the autocorrelation method for estimating the standard uncertainty of an observable. The essential objective of this technique is to estimate the number of independent samples in a simulation, via an intermediate function known as the autocorrelation function. First, given a set of observations $\{q_1, ..., q_N\}$, the autocorrelation function *C* is computed for a set of lags *j*:

$$C_j = \frac{\overline{\left(q_k - \bar{q}\right)\left(q_{k+j} - \bar{q}\right)}}{s\left(q_k\right)^2} \tag{11}$$

NOTE: Have to decide how to express an average here; we reserved <· · ·> for true expectation values.

where the denominator is the square of the experimental standard deviation of q_k given by Eq. 6. Then, the number of independent samples is estimated by[8]

$$N_{ind} = \frac{N}{1 + 2\sum_{i=1}^{N_{lags}} C_j} \tag{12}$$

where N_{lags} is the number of lags for which the C_j was computed. In practice, one rounds N_{ind} to an integer. Finally, the standard uncertainty is estimated via

$$s\left(\bar{q}\right) = \frac{s\left(q_k\right)}{\sqrt{N_{ind}}} \tag{13}$$

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[8]Explain why this sum is not the same as the integration over *C*; *C* is stationary and symmetric, 1 comes from the "zeroth" lag, 2 is for symmetry. Actually easy to see this if you do an integral from -infty to +infty by a midpoint integration.

We note that the experimental standard deviation of the observable *q* is used in Eq. 13 to estimate the uncertainty. Strictly speaking, the standard uncertainty should be estimated using the true standard deviation of *q* (e.g. σ_q); given that the true standard deviation is unknown, the experimental standard deviation is used in its place as an *unbiased estimate* of σ_q (GOING TO NEED A CITATION HERE!!!).

7.5 Propagation of uncertainty

The quantities we are most interested in may not be simulation observables. For instance, the free energy difference between two states might be measured by free energy perturbation, expressed as a function of the average of another quantity [41].

$$\beta\Delta A = -\ln\left\langle\exp\left(-\beta\Delta U\right)\right\rangle \tag{14}$$

Although $\exp(-\beta\Delta U)$ can be measured during the simulation and its uncertainty can be estimated directly using block averages as described above, $\beta\Delta A$ cannot be handled the same way. If we compute $\beta\Delta A$ for each block, the values will tend to take extremely positive whenever the perturbation does poorly (where ΔU is consistently large). In the pathological case, ΔU might be ∞ for every sample in a block and the $\beta\Delta A = -\ln 0$ cannot be computed.

Instead of using block averages for $\beta\Delta A$, its uncertainty can be expressed as a first-order Taylor series expansion

$$\sigma_{\beta\Delta A} = \sigma_{\exp(\beta\Delta U)}/\left\langle\exp\left(-\beta\Delta U\right)\right\rangle \tag{15}$$

Propagation of uncertainty is needed whenever the derived quantity can be expressed as a function of other random observables. It might also be needed when the derived quantity is of a function of quantities measured in separate simulations, such as $< U(T_2) > - < U(T_1) >$. If a derived quantity is a function of multiple observables measured within a single simulation, then terms must be included to account for the correlation between those observables.

The Taylor series approach works well in most cases and is easy to use, but does have limitations. Because this approach is based on a first-order Taylor series, propagation of uncertainty can fail in cases where a non-linear formula is used and the uncertainty is very large or the distribution of input averages is not Gaussian. For instance, the uncertainty in $\beta\Delta A$ as prescribed by Eq. 15 cannot exceed unity no matter how short the simulation is or how bad the sampling is. If there is doubt as to the quality of the computed uncertainty, the uncertainty can be estimated with alternative approaches such as bootstrapping to validate the Taylor series results or to identify an alternative approach that works better.

n (independent samples)	k (coverage factor)
6	2.57
11	2.23
16	2.13
21	2.09
26	2.06
51	2.01
101	1.98

Table 1. Coverage factors k required for a two-sided 95 % confidence interval for a Gaussian variable [19].

7.6 From standard uncertainty to confidence interval for Gaussian variables

Once a standard uncertainty value is obtained for a Gaussian-distributed random variable with mean $\langle x \rangle$, and the number of independent samples n has been estimated, the 95 %-confidence interval $(\bar{x} - k\,s(\bar{x}), \bar{x} + k\,s(\bar{x}))$ can be constructed on the basis of an established look-up table (or a statistics software model) for the coverage factor k based on n. The theoretical basis for the table is the "Student" or "t" distribution, which is *not* Gaussian, but governs the behavior of an *average* derived from n independent Gaussian variables [19]. Table 1 lists k for two-sided 95 % confidence intervals for select values of n.

When $n \leq 10$, we recommend showing all data points, as a confidence interval may not be statistically meaningful.

As a reminder, multi-modally distributed variables with multiple peaks in their distributions cannot be considered Gaussian random variables. Variables with a strict upper or lower limit (such as a positive-definite quantity) and long-tailed distributions are also not Gaussian. These cases should be treated with bootstrapping.

7.7 Bootstrapping

Bootstrapping is an approach to uncertainty estimation that does not assume a particular distribution for the observable of interest or a particular kind of relationship between the observable and variables directly obtained from simulation [11]. In nonparametric bootsrapping, new, "synthetic" data sets (corresponding to hypothetical simulation runs) are created by drawing n samples (configurations) from the original collection that was generated during the actual run. The same sample may be selected twice, while others may not be selected at all in a process called "sampling with replacement." In doing so, these synthetic sets will be different even though they all have the same number of samples and draw from the same pool of data. Having created a new set, the data is analyzed to determine the derived quantity of interest, and this process is repeated to produce multiple estimates of the quantity. The distribution of 'synthetic' observables can be directly used to construct a 95 % confidence interval from the 2.5 %ile to the 97.5 %ile value.

The process described above assumes that the original simulation data is uncorrelated. If this is not the case, then the resampling method can be reformulated in one of two ways. The first option is to estimate the number of independent samples in the original set (e.g., using an autocorrelation method [7, 24]) and to pull only that many samples to create the new data sets. The second option is to group the samples into blocks that are uncorrelated based on analyzing varying block sizes (see above) and to then use the block averages as the samples for bootstrapping.

Alternatively, one could use the difference between errors estimated via block averaging and bootstrapping as a measure of the correlation; if one tracks the bootstrapped and block averaged estimates of a quantity's uncertainty as a function of block size, the only difference between the two modes of calculation is whether the data is correlated. The decay in the ratio of the two quantities as a function of time is a measure of the correlation time in the sample [35].

7.7.1 Bootstrapping variants

An alternate approach that can directly account for correlations is called parametric bootstrapping. The main idea behind this method is to model the original data as a deterministic function (which can be zero, constant, or have free parameters) plus additive noise. The parameters of this model, including the structure of the noise (i.e. its covariance), can be determined through a statistical inference procedure. Having calibrated the model, random number generators can be used to sample the noise, which is then added back to the trial function to generate a synthetic data set. As with the nonparametric bootstrap, the generated data can be used to compute the derived quantity of interest, and the uncertainty can be obtained from the statistics of the values compute with different generated sets.

To further clarify the procedure of parametric boostrapping, consider the simplest case in which the data is a collection of uncorrelated random variables fluctuating about a constant mean. In this situation, one could estimate (I) the deterministic part of a parametric model using the sample mean $\bar{q}$ of the data, and (II) the stochastic part as a Gaussian random variable whose variance equals the sample variance. If instead the data are correlated (e.g. as in a time-series of simulated observables), one can postulate a covariance function to describe the structure of this randomness. Often these covariance functions are formulated with free parameters (often called "hyperparameters") that characterize properties such as the noise-scale and characteristic length of correlations [31]. In such cases, determining the hyperparameters

may require more sophisticated techinques such as maximum likelihood analyses or Bayesian approaches; see, for example, Ref. [31]. See also Refs. [4, 5, 26, 27] for examples and practical implementations applied to cases in which the determinsitic component of the data is not constant.

An alternative to bootstrapping is the "jackknife" method[29, 30, 42] (also outlined in Ref. [11]). It operates similar to the bootstrap as a resampling technique, but it uses synthetic data sets created by subtraction of samples rather than replacement; as such it is often categorized as a variant of the bootstrap (even though it predates the bootstrap). Since it operates by sample deletion, it may be better suited to smaller data sets for which a few samples may be overrepresented in the synthetic data set created by the bootstrap replacement technique. Ultimately, though, the results are similar in that the jackknife technique creates a distribution of derived observables that can be used to compute both an arithmetic mean and estimate of the standard uncertainty.

7.7.2 Bootstrapping and uncertainty propagation

It is important to note that various bootstrapping approaches can and often are used as uncertainty propagation tools. Nonetheless, care should be exercised when using such methods with nonlinear functions. In the free energy example, setting $\langle \exp(-\beta\delta U) \rangle = 1 \pm 0.5$ and generating new estimates from a Gaussian centered at 1 with a width of 0.5 will eventually output negative numbers, which is mathematically nonsensical and problematic for any function that takes strictly non-negative inputs. Thus, one should be aware of any distributional assumptions imposed either by the physics of the problem or the analyses of synthetic data.

7.8 Dark uncertainty analyses

In some cases, multiple simulations of the same physical observable τ may yield predictions whose error bars do not overlap. This situation can arise, for example, in simulations of the glass transition temperature when undersampling the crosslinked network structure of certain polymers. In such cases, it is reasonable to postulate an unaccounted for source of uncertainty, which we colorfully refer to as "dark uncertainty." In the context of a statistical model, we postulate that the probability of a simulation output depends on the unobserved or "true" mean value $\bar{\tau}$, an uncertainty σ_i^2 whose value is specific to the simulation (estimated, e.g. according to uncertainty propagation), and the unaccounted-for dark uncertinty y^2. (For simplicity, the σ_i^2 and y^2 should be treated as variances.)

While details are beyond this scope of this document, such a model motivates an estimate of $\bar{\tau}$ of the form

$$\bar{\tau} \approx \mathcal{T} \propto \sum_i \frac{T_i}{\sigma_i^2 + y^2}, \tag{16}$$

where T_i is the prediction from the ith simulation, σ_i^2 is its associated "within-simulation" uncertainty, and y^2 is the dark or between-simulation uncertainty; note that the latter does not depend on i. The variable y^2 can be estimated from a maximum-likelihood analysis of the data and amounts to numerically solving a relatively simple nonlinear equation (see Ref. [26]). Equation (16) is useful insofar as it weights simulated results according to their certainty while reducing the impact of overconfident predictions (e.g. having small σ_i^2). Additional details on this method are provided in Ref. [26] and the references contained therein.

8 Assessing Uncertainty in Enhanced Sampling Simulations

While recent advances in computational hardware have allowed MD simulations of systems with biological relevance to routinely reach timescales ranging from hundreds of ns to µs, in many cases this is still not long enough to obtain equilibrated (i.e. Boltzmann-weighted) structural populations. Intrinsic timescales of the systems may be much longer. Enhanced sampling methods can be used to obtain well-converged ensembles faster than conventional MD. In general, enhanced sampling methods work through a combination of modifying the underlying energy landscape and/or thermodynamics parameters to increase the rate at which energy barriers are crossed along with some form of reweighting to recover the unbiased ensemble [46]. However, such methods do not guarantee a converged ensemble, and care must be taken when using and evaluating enhanced sampling methods.

Generally speaking, uncertainty analysis is more challenging for data generated by an enhanced sampling method. Before performing such a simulation, consider carefully whether the technique is needed, and consult the literature for best practices in setting up a simulation. Even a straightforward MD simulation requires considerable planning, and the complexity is much greater for enhanced techniques.

8.1 Replica Exchange Molecular Dynamics

One of the most popular enhanced sampling methods is replica exchange MD (REMD) [39]; see also [40]. Broadly speaking, REMD consists of running MD simulations a number of non-interacting replicas of a system, each with a different Hamiltonian and/or thermodynamics parameters (e.g., temperature), and periodically exchanging system coordinates between replicas according to a Metropolis criterion.

In order to assess the results of a REMD simulation, it is important to consider not just the overall convergence of the simulation to the correct Boltzmann-weighted ensemble of structures (via combined clustering, combined PC projection overlap analysis, etc.), but how efficiently the REMD simula-

tion is doing so. These concepts are termed "thermodynamic efficiency" and "mixing efficiency" by Abraham and Gready,[1] and it is quite possible to achieve one without the other; both must be assessed. In order for sampling to be efficient, coordinates must be able to move freely in replica space.

Below we will refer to both "coordinate trajectories" and "replica trajectories". A "coordinate trajectory" follows an individual system's continuous trajectory as it traverses replica space (e.g., a system experiencing multiple temperatures as it is exchanged during a temperature REMD simulation). A "replica trajectory" is the sequence of configurations corresponding to a single replica under fixed Hamiltonian and thermodynamic conditions, (e.g., all structures at a temperature of 300 K in a temperature REMD simulation). Thus, a replica trajectory consists of concatenated coordinate-trajectory segments and *vice versa*.

- Exchange acceptance. The exchange acceptance rate (i.e. the number of exchanges divided by the number of exchange attempts) between neighboring replicas should be roughly equivalent to each other and to the target acceptance rate. If the exchange acceptance is too low then replica spacing may need to be decreased or additional replicas used, and vice versa if the exchange acceptance is too high.

- Replica round-trips. The time taken for a coordinate trajectory to travel from the lowest replica to the highest and back is called the replica "round trip" time. Over the course of a REMD simulation, any given coordinate trajectory should make multiple round trips. In addition one can look at the average, minimum, and maximum round trip times: these should be roughly equivalent for any given set of coordinates. See e.g. Figure 6 in [32].

- Replica residence time. The time coordinate trajectory spends at a replica is called the "replica residence time". For replica sampling to be efficient, the average replica residence time for each set of starting coordinates at each replica should be roughly equivalent. See e.g. Figure 7 in Roe et al..[32]

- Distributions of quantities calculated from coordinate trajectories. If all coordinates are moving freely in replica space, they should eventually converge to the same ensemble of structures. Comparing distributions of various quantities from coordinate trajectories can provide a measure of how converged the simulation is. For example, one can compare the distribution of RMSD values of coordinate trajectories to a common reference structure; see e.g. Figure 8 in Henriksen et al..[16] Poor overlap can be an indication that replica efficiency is poor or the simulation is not yet converged.

All of the above quantities (replica residence time, round trip time, lifetimes etc) can be calculated with CPPTRAJ,[33] which is freely available from https://github.com/Amber-MD/cpptraj or as part of AmberTools (http://ambermd.org).

It may also be useful to perform multiple REMD runs. Using the standard uncertainty among runs can quantify uncertainty and provide the basis for a confidence interval with an appropriate coverage factor - see definitions in Sec. 1. If the ensembles produced depend significantly on the set of starting configurations, that is a sign of incomplete sampling.

8.2 Weighted Ensemble simulations

The weighted ensemble (WE) method orchestrates an ensemble of trajectories that are intermittently pruned or replicated in order to enhance sampling of difficult-to-access regions of configuration space [18]. The final set of trajectories can be visualized as a tree structure based on the occasional replication and pruning events. WE is an unbiased method that can be used to sample rare transient behavior [44] as well as steady states [3] including equilibrium [37].

Like other enhanced sampling methods, WE's tree of trajectories has a complex correlation structure requiring care for uncertainty analysis. It is important to understand the basic theory and limitations of the WE method, as is discussed in a WE overview document.

From a practical standpoint, the safest way to assess uncertainty in WE simulations is to run multiple instances (which can be seeded from identical or different starting structures depending on the desired calculation) from which a variance and standard uncertainty in any observable can be calculated; see definitions. Note particularly that WE tracks the time evolution of observables as the system relaxes (perhaps quite slowly) to equilibrium or another steady state [44]; hence, the variance computed in an observable from multiple runs should be based on values at the same time point.

When it is necessary to estimate uncertainty based on a single WE run, the user should treat the (ensemble-weighted) value of an observable measured over time much like an observable in a standard single MD simulation; this is because the correlations in ensemble averages are sequential in time. First, as discussed in Sec. 4, the time trace of the observable should be inspected for relaxation to a nearly constant value about which fluctuations occur. A transient/equilibration period should be removed in analogy to MD - see Sec. 5 - and then best practices for single obervable uncertainties should be followed as described in Sec. 7. Despite this rather neat analogy to conventional MD, experience has shown that run-to-run variance in WE simulations of challenging systems can be large, so multiple runs are advised. In the future, variance-reduction techniques may alleviate the need for multiple runs.

9 Acknowledgments

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