The linac coherent light source single particle imaging road map

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Intense femtosecond x-ray pulses from free-electron laser sources allow the imaging of individual particles in a single shot. Early experiments at the Linac Coherent Light Source (LCLS) have led to rapid progress in the field and, so far, coherent diffractive images have been recorded from biological specimens, aerosols, and quantum systems with a few-tens-of-nanometers resolution. In March 2014, LCLS held a workshop to discuss the scientific and technical challenges for reaching the ultimate goal of atomic resolution with single-shot coherent diffractive imaging.

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This paper summarizes the workshop findings and presents the roadmap toward reaching atomic resolution, 3D imaging at free-electron laser sources.
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I. INTRODUCTION

One of the grand visions for X-ray free-electron lasers (XFEL) is the ability to image non-repetitive and non-reproducible structures with single femtosecond x-ray pulses.[1] The underlying concept is that the x-ray pulses are so intense that the imaging process is outrunning the sample damage.[2] First single particle imaging (SPI) experiments performed at the Linac Coherent Light Source (LCLS) have been very successful, producing single-shot coherent diffraction images of viruses,[3] bacteriophages,[4] organelles,[5] and cyanobacteria[6] to name a few. A variety of other biological structures have been imaged with the goal of creating data sets for algorithm development.[4] First steps have been taken to assemble single shot images into three dimensional data sets.[7] In addition to biological systems, single shot diffractive imaging has been used to study the morphology of aerosols,[8,9] power density dependent damage processes in atomic clusters,[10] and superfluid quantum systems.[11]

The resolution of single-shot single-particle imaging experiments at LCLS has so far been limited to a few tens of nanometers. To unlock the full potential of imaging experiments with free-electron lasers, including the ability to perform time-resolved studies, atomic resolution needs to be achieved. But currently, it is not clear if atomic resolution from single particles can be reached and if so, which technical and scientific problems have to be solved. In this spirit, LCLS has hosted a by-invitation workshop with renowned experts in the field of ultrafast radiation damage, imaging algorithm development, XFEL instrumentation, and sample issues. The charge to the workshop attendees was to "Define an R&D roadmap to achieve 3D atomic-resolution single-particle imaging using X-rays" with a focus on the following questions:

- What pulse characteristics are required?
- Can we overcome radiation damage by XFEL pulses?
- What are the advantages and disadvantages in comparison with competing approaches such as cryo-electron microscopy (cryo-EM)?

The workshop was organized with alternating plenary and break-out sessions for best possible overlap and discussions between the various working groups. The overwhelming consensus of the workshop attendees was that single particle imaging with atomic resolution at free-electron lasers is in principle feasible but that a variety of technical and scientific issues have to be solved. A road map aimed at achieving single-particle imaging at free-electron lasers with atomic resolution has been formulated. This paper summarizes the workshop findings and presents the road map.

II. RADIATION DAMAGE ON THE FS TIME SCALE

A. Our current picture of radiation damage

Any sample in single-shot imaging experiments will undergo dynamic changes during the duration of the x-ray pulse. The samples will be efficiently photo-ionized in the photon energy regime between 1 keV and 10 keV as, in particular, the core-electron absorption cross section is significantly larger than the scattering cross section. For the case of carbon, the absorption will be saturated at 1 keV for fluences of 10^9 photons/$(100\,\text{nm})^2$ and this number increases to 10^{12} photons/$(100\,\text{nm})^2$ at 8 keV.[12]

In the x-ray regime accessible today, the atomic photo ionization processes are well described with sequential absorption,[13] and it is expected that this holds true for power density increases up to at least three orders of magnitude compared to the currently available power

density regime. The photoelectrons will very quickly deposit further energy into the sample by inelastic collisions and lead to secondary ionization processes.[14] A fast photoelectron with a kinetic energy of 8 keV travels on the order of 45 nm per femtosecond, but its mean free path is only on the order of 4 nm. Inner-shell vacancies will be filled with valence electrons either by Auger or fluorescence decays. For low Z elements, Auger decay dominates and typical Auger lifetimes are on the order of ten femtoseconds. The electrons, and here, in particular, the secondary and Auger electrons, are efficiently trapped in the building Coulomb potential of the sample and form a plasma. The energy transport in such a plasma cannot yet be completely described,[15] and it is noted that the plasma environment can significantly alter the sample ionization dynamics.[16] Ultimately, the sample will disintegrate depending on its size and chemical composition in a Coulomb explosion or hydrodynamic expansion[10] which so far can be only modeled for small samples.

The ionization processes described above will alter the imaging response of a biological sample.[2] The atomic form factors, which describe the scattering response of the sample, will change during the x-ray pulse reducing the contrast in single-particle diffraction data.[17] Further, the contributions from inelastic scattering and scattering from free electrons exceed the elastic scattering strength at incident intensities of 10^{14} photons/(100 nm)^2 and scattering beyond 4 Å.[18]

B. Resulting beam parameters

Our current understanding of radiation damage implies that the most important measure to limit its effect is to shorten the x-ray pulse. Although initial photoionization and subsequent secondary electron creation cannot be circumvented, pulses shorter than typical Auger lifetimes, i.e., in the few-femtosecond regime, can maximize the scattering contrast by avoiding Auger recombination and cascade processes within the pulse duration. In terms of photon fluences, the order of 10^{12}–10^{13} photons/(100 nm)^2 appears most attractive for optimal signal contrast of delivered photons.

While a definite recommendation on the photon energy cannot be made right now, we are convinced that all essential physical processes are sufficiently known for making systematic simulations possible in the near future. We believe that for the goal of 3 Å resolution and the technological/geometric limits of detectors, the 3–8 keV range seems suitable for initial tests aimed at studying damages at high resolution.

C. Atomic, molecular, and optical physics tricks for controlling radiation damage

Tamper layers have been proposed to supply electrons to the ionized sample and to confine the geometry of the single particle by delaying its Coulomb explosion.[19] While use of a tamper layer is conceivable for conceptually simple systems, its use for more complex and biologically relevant systems is difficult and deemed to not be a likely path to success toward imaging at 3 Å. Further, in imaging experiments, the tamper layer has to be thin in order to keep the scattering background from the tamper low. Macromolecules typically have a mixture of hydrophobic and hydrophilic surfaces and the wetting of these surfaces by a thin layer of water is not uniform and the tamper layer becomes dysfunctional. In addition, the tamper layer has to be identical around each macromolecule to avoid incoherent contributions to reconstructions.[20] The most important bio-compatible tamper material is water, which becomes disordered in thicker layers.

Molecular alignment by electromagnetic trapping was discussed as a means to obtain additional information about single particle orientation. While this method had some recent success for small molecules,[21] it is currently unclear if the application of electromagnetic fields leads to the deformation of large molecules. While larger molecules with high aspect ratios have an increasing anisotropy of the polarizability tensor and are thus easier to align compared to diatomic molecules, they are also characterized by very shallow binding potentials, enhancing the probability for damage. More systematic research is appropriate on this point.

D. Conclusions, unknowns, and the way ahead

The working group came to the conclusion that there appears to be no fundamental road block for single particle imaging. While the sample will always be damaged by the x-ray pulse, i.e., strong ionization and changing scattering factors are unavoidable, there is belief that the underlying physical processes are known in sufficient detail. However, many more experiments and simulations are required to fully understand all phenomena at play in complex molecules. Modeling the damage is thought to be feasible and we are optimistic that the so-obtained information could be used to reconstruct sample details. Therefore, obtaining structural information from single particle imaging experiments appears possible. However, concerns exist about obtaining electron density details, such as detailed bonding configuration.

The working group identified a few topics that are beyond current knowledge and need further investigation:

- Most modeling is based on atomic assumptions, how do molecular effects change the picture? How do chemical effects such as bond-breaking effect the damage mechanisms and what is the role of localized damage?
- What are the mechanisms of energy transport in highly excited, confined systems? What is the role of charge migration during the x-ray pulse?
- Will it be possible to recover undamaged electron density maps based on damaged sample data?
- Does the current picture of single photon scattering hold in the limit of extreme intensities?
- For very short pulses, how is the scattering response of the changing sample related to the static electron distribution?

In order to tackle these questions and to develop a complete picture of radiation damage at high x-ray intensities, the working group proposes the following next steps:

- Evaluate the optimal photon energy by simulations taking realistically achievable instrumental resolution, damage processes, and signal strength into account.
- Benchmark the damage models experimentally with systems of increasing complexity.
- Critically question theoretical assumptions.
- Investigate if modeling undamaged electron density maps based on dynamically ionized sample data is possible.
- Engage in experiments to understand transition rates, electron recapture, and the effect of local environments on damage processes in large molecules.

III. ALGORITHMS: IMAGE ORIENTATIONS AND 3D RECONSTRUCTION

Imaging single, reproducible particles using an XFEL involves an inverse problem, whose solution requires both physical insight and algorithm development. Coherent diffractive imaging (CDI), a well-developed technique for storage rings,[22] allows the retrieval of the 3D structure of the sample of interest from its continuous 3D diffraction data. The extension of this to XFEL data is, in parts, both trivial and challenging.

The highly coherent x-ray beam produced by an XFEL provides CDI data much closer to the assumptions made in CDI. Therefore, the inversion algorithms[23] that lie at its core are, in general, even more effective at converting continuous diffraction data into structural information. However, the highly coherent nature of the x-ray source is far from the only difference between the single-particle imaging problem and the traditional, storage ring-based CDI applications.

A. The orientation challenge

One novel aspect of the XFEL single-particle imaging problem is that the same burst of x-rays that provides a single projection of the sample almost immediately destroys it. Since a 3D structure is desired, information from different projections of many identical copies of the

sample must be integrated. The alternative of injecting hydrated biomolecular particles of accurately known orientation appears to be impractical. We are, therefore, left with the challenge of algorithmically determining the orientations of successive particles in order to assemble a 3D data set.

This problem is well known within the community, and several research groups have demonstrated algorithms[24,25] capable of assembling 3D diffraction volumes from simulated random snapshots at signal levels expected from single ($\sim$500 kD) biomolecules with Poisson noise, and, in some cases Gaussian background scattering. Indeed, simulations have shown that snapshots containing only a few scattered photons per frame suffice from which phasing algorithms can recover the macromolecular structure. The current status is that 3D reconstructions of particles at modest resolution have been achieved.[7] Although 2D data sets from viruses exist, their assembly into 3D remains a work-in-progress.

The general availability of "standard" experimental data sets, either from biomolecular single-particles or those closely resembling such snapshots would considerably facilitate the optimization of experimental and theoretical techniques, and, thus, is of high importance. Since inhomogeneity increases with size in nature, the choice of a standard test object will require careful consideration. We recommend that these studies proceed in a pre-competitive and cooperative environment.

B. The signal challenge

In most cases, the samples of interest in a single-particle imaging study are weakly scattering—e.g., biomolecules—which leads to low signal-to-noise ratios in the collected data. Further complicating this, sample heterogeneity poses a significant risk to the correct assembly of a complete data set. This problem is known from single particle cryo-EM. Realistic estimates of heterogeneity and algorithmic studies are needed as a matter of urgency to assess the scope of this problem. To complicate the matter even further, XFEL sources have significant shot-to-shot fluctuations resulting from the self-amplified spontaneous emission process, making the heterogeneity of the collected data even worse, with both the sample and the beam contributing to fluctuations in the collected signal.

It is of vital importance to the community as a whole to have access to multiple shared data sets, sufficient for both orientation and structure determination, and to possess a forum for the comparison and competition of algorithms and approaches. We recommend the evaluation of a "round robin" testing program, where multiple blind trials are conducted by different groups on the community's shared data sets. Data sets should be open for download by anyone to encourage new algorithmic approaches.

C. The noise challenge

The greatest impediment to the success of a single-particle imaging experiment is the various background noise introduced into the measured signal from "extraneous" experimental factors, such as shot-to-shot variations in the beam position and intensity, and scattering from apertures and particle-injection beams. Simulations have shown that the introduction of certain types of noise, for example, Poisson noise and additive background Gaussian noise, pose less risk to the successful application of orientation and inversion algorithms. Extraneous noise, most notably that arising from the optical elements along the instrument's beam path and the means used to inject hydrated particles into the x-ray beam when using a water jet, pose a major challenge, because it dominates the correlations between snapshots, which form the basis of any orientation recovery algorithm. The successful resolution of this challenge will involve direct communication among the developers of optics, instruments, including sample delivery, and data analysis.

It is vitally important that every reasonable instrumental measure be taken to minimize scattered photons from "particle-extraneous" factors. At their current level, these factors prevent existing algorithms from achieving reliable, biologically relevant 3D reconstructions. We

therefore believe new experimental and algorithmic approaches must be urgently developed and validated to mitigate the impact of "extraneous" scattering so as to extract reliably integrated data.

As new sample delivery techniques are established, corresponding standard, state-of-the-art data sets must be made available for testing and fine-tuning of data processing and 3D structure-recovery algorithms. Since these are likely to depend strongly on the instrument configuration, we recommend that an effort be made to develop, achieve, and maintain a set of standard instrument configurations for single-particle experiments.

In the same way that x-ray scattering from sources extraneous to the sample limits our ability to identify signals that contain structural information, we rely heavily on calibrated and "well-behaved" x-ray detectors. These detection systems should provide single-photon sensitivity, minimal cross-talk, and exceptional linearity over a wide dynamic range. We recommend a robust effort in detector development, characterization, and calibration. Such an effort must be guided by simulation to determine the required detector characteristics, and in practice, by what can be achieved in a real experiment.

It is our opinion that the community's library of algorithms for modeling radiation damage is sufficient for the data treatment and analysis—in the absence of novel damage mechanisms and extreme sample heterogeneity.[14,26] However, we foresee that significant new efforts will be required at the interface between the conceptual and the actual experiment.

D. The sample heterogeneity challenge

Identical objects are almost never identical, particularly at high resolution. Indeed, biologically important functions proceed via structural and conformational changes, and are of substantial scientific interest. Most orientation and structure recovery approaches are predicated on sightings of identical objects in different projections. It is imperative that algorithms be further developed[5,27] to deal naturally and efficiently with sample heterogeneity, and specify the number and quality of snapshots needed to extract reliable information from heterogeneous datasets.

IV. BEAM CHARACTERISTICS, BEAMLINE INSTRUMENTATION

The beam characteristics, beam line instrumentation breakout group of the Single Particle Imaging Workshop focused on the required hardware and a procedural road map to realize single particle imaging. Specific focus was given to determining the desired beam parameters, the needed steps in development of accelerator and X-ray beamline instrumentation, required detector parameters, and an experimental roadmap for the required technical developments.

A. Desired beam parameters

To realize the goal of single molecule imaging with near atomic resolution of 3 Å, the working group came to conclusions on the FEL photon beam size, focal spot size, and wave front parameters. With input from the other working groups, a target photon energy range of 3 keV to 8 keV was identified as most suitable. This photon energy range provides flexibility in operation and the desired resolution of 3 Å while still keeping a forward scattering geometry. In addition, limiting the photon energy range to a factor of order 2 potentially can limit the harmonic content. The working group feels that if the working photon energy range was extended beyond this target range, two optimized beamlines would be better suited then a single beamline.

The 3 to 8 keV photon energy range also brings with it challenges to the optics design. As x-ray compound refractive lenses are strongly absorbing below 5 keV, the only viable focusing elements are Kirkpatrick Baez (KB) grazing incidence mirrors. Such mirrors can provide high quality focal spots while reducing the harmonic content. The group feels that what drives the need for a small focus is not the spot size itself but rather the power density and "clean beam" required to obtain a measurable and interpretable signal. During the discussion with the other working groups, a focal spot size between 100 and 200 nm was perceived as necessary. While

it is recognized that a larger, micron-sized focus can provide useful scientific advancements, overall more photons are needed on the sample for the goal of 3 Å resolution. This could be achieved by more intense X-ray pulses or a smaller, more intense focus due to the typically small size of objects reproducible at the 3 Å level. The report however notes contradictory opinions stating that having the tightest possible focus may not be the best strategy, if doing so introduces strong "halos" around the beam that also fluctuate in time. Increasing the signal is desirable, but not at the cost of introducing too many unknowns in the modeling of the background. The existing debate on the best focus to use is only one of many such debates regarding ideal beam parameters and will be worth exploring experimentally.

Of additional importance is a flat wave-front in the focal plane and the ability to position the sample within the Rayleigh length of the focal plane. This is a challenge and requires improvements in diagnostics and minimal added distortions along the FEL beam. Thorough characterization of the current FEL focus is required and will help guide future optics designs.

B. Accelerator and X-ray beamline instrumentation

A general discussion with all four working groups came to the conclusion that pulse lengths need to be <20 fs—preferably even less than the Auger lifetimes of 10 fs—and each pulse ideally would contain up to 10^{14} photons to produce reasonable signal levels. It is noted that the FEL bandwidth is not a limitation in single particle imaging due to the typical number of resolution elements in the images, that is, the ratio of the resolution to the object size. This ratio does not usually exceed 100, which is less than the ratio of photon energy to bandwidth. Only for higher ratios and thus, very high resolution, eventually the bandwidth may become a limitation. The number of resolution elements is limited to the inverse of the bandwidth due to different wavelengths arriving out of phase. Therefore, producing the highest number of photons should be prioritized over bandwidth control. With space-charge limiting the number of electrons in a femtosecond electron bunch, increasing the number of photons produced by each electron in the bunch is desirable. To achieve this goal, the working group endorses the developmental research on superconducting undulators[28] to increase photon yield in the fundamental and potentially decrease harmonic content.

With these accelerator developments come an equal need for improvements and upgrades on the photon beam transport and beamline design. Beamline scattering is of particular concern, as scattering from the beamline optics can easily swamp scattering from the sample. Optical design and material selection must be performed so as to make every effort to reduce beamline scattering as far as possible. Specifically, windowless operation was discussed at length and the need for a differential pumping section between the optics and the focus. Novel "window" designs were discussed to provide a physical barrier to mirror contamination and obviate the need for differential pumping, but the R&D path toward this goal is unclear. The group feels that if a compromise is required between the focal spot size and windowless operation, providing a larger focal spot would be the most effective option, consistent with the opinions of others expressing that more signal is not always better if it comes with more noise. In addition, diagnostics for vibrational control of the optics would allow the jitter and drift of the focal plane to be monitored and potentially allow implementation of feedback on the position of the focal plane. Perpendicular sample viewing for focal plane and sample injection overlap would be beneficial to maintain the sample at the optimal focus.

The distribution system and beamlines need to support focusing of 3–8 keV photon energies, most likely via KB mirrors. This will require long front-end mirrors at steep angles to support the larger, more divergent, lower photon energy x-ray beams. Larger focusing optics will also be required, or alternatively the construction of a new hutch closer to the source. In addition, the working group recommends the development and testing of various slits and aperture designs. This is due to the fully coherent nature of the FEL and that any component in the low intensity tail of the FEL can lead to significant and structured scattering.

C. Detector development

The list below summarizes the desired specification in the priority order:

- A photon energy range of 3–8 keV with high quantum efficiency over the full range.
- Single photon counting ($>7\sigma$) at high q and lower than Poisson noise elsewhere.
- A dynamic range of at least 10^4 is desired. We endorse a hybrid solution as intensity will fall off with increasing angle.
- Pixel size of 100 μm or smaller. This is needed to make the experimental systems manageable in size.
- A readout speed of 1 kHz, with a desired ultimate speed of 10 kHz.
- Good vacuum operation capability.
- A form factor of 2 k by 2 k pixels or greater.

V. SAMPLE ISSUES: DELIVERY, HETEROGENEITY, BIOLOGICAL SIGNIFICANCE AND OTHER STRUCTURAL METHODS

A. Biological significance and other structural methods

The ultimate impact and niche of CDI must be considered in the context of recent developments in cryo-EM analysis of single particles. cryo-EM is limited in scattered flux per exposure and in temporal resolution. Sample thickness is also limited by multiple inelastic scattering, and thick biological samples typically have significant heterogeneity in structure. Recent developments, in particular, direct electron detectors with high sensitivity and frame rates that allow computational correction of beam-induced specimen movement have enabled determination of structures at 3–4 Å resolution, in the range of 200 KDa to several MDa particles. It is anticipated that within a few years achieving 3 Å resolution for particles as small as 50 KDa will be possible by cryo-EM. Nonetheless, a potential synergy could be to use CDI data to extend the resolution of a low-resolution cryo-EM map.

Cryo-electron tomography has enabled structural analysis of thick samples including large viruses, organelles, and larger sections of cells. Because a single particle is examined and reconstructed from images at different tilt angles, heterogeneity can be readily detected by comparing different specimens. The resolution of this method is low, however, due to the low electron doses needed to avoid sample damage. Nonetheless, many large biological objects are heterogeneous (non-reproducible), so it is not clear whether there are high-resolution questions that can be addressed by CDI for samples too large for single-particle EM. If there are, then CDI clearly offers an advantage.

CDI "freezes" motion at room temperature by using ultrashort and extremely bright coherent X-ray pulses and has potential advantages over cryo-EM in several other areas. There is no background from vitreous ice or from a sample holder. The ability to examine particles at ambient temperature could be very important, as it is known that some biological samples can be altered by the surface tension of the vitreous ice. Second, for time-resolved studies, for example, examining conformational changes following a triggered reaction, cryo-EM is limited to tens of ms by the sample preparation process—essentially how quickly a sample can be plunge-frozen following triggering the reaction of interest. Many conformational changes of interest occur in the ns-ms range, in particular, the larger-scale changes involving movements of domains and secondary structure elements, so time-resolved studies by CDI would be advantageous.

B. Sample heterogeneity

Sample heterogeneity is an impediment to achieving high resolution in both CDI and cryo-EM. Although heterogeneity arising from sample preparation can be minimized by careful biochemical characterization, many systems are inherently dynamic and must thus be dealt with at the data analysis stage. Ongoing improvements in classification algorithms will be essential, but

even when a sample can be binned into a small number of conformational states, there is often debate as to practical limits on the number and relevance of states. By overcoming the damage limit, CDI may provide some clarity into this problem.

C. Sample delivery

It is currently possible to collect CDI data at reasonable rates, but contaminants and sample consumption are problematic. Producing droplets with average size below 200 nm is currently difficult. This implies either that the object of interest must be imaged while in a droplet much bigger than the sample, with consequent poor signal-to-noise, or that water is removed and all remaining materials that do not evaporate remain on the particle, resulting in inhomogeneous size distributions that reflect the initial droplet size distribution. Such situation likely prohibits 3D reconstruction due to the poor contrast between "crust" and particle. The delivery of the sample in vacuum and the subsequent removal of water can also potentially produce a denatured sample. Sample injection methods typically move the sample at speeds of tens to hundreds of meters per second, moving large amounts of sample past the interaction region between shots. Consequently, a large amount of sample is needed, in the range of 10^{14} particles for a complete data set in some cases.[29]

Several alternatives were discussed. Cryo-sheets of sample, essentially those used for cryo-EM, might be possible, but of course this removes the advantage of working at ambient temperature. Capture in ~30 nm micelles was proposed as means of keeping the molecule in its native state while in a smaller droplet. It was felt that it would be useful to explore modified high throughput electrospray injection methods, in which the sample is kept in its native environment for as long as possible before removing water and buffer components when injected into the beam. Given the timescales of protein denaturation, it was estimated that the detrimental effects of removing water and salts will probably occur on timescales longer than microseconds.

D. Recommendations

CDI has an advantage over cryo-EM in being able to study thick and room temperature samples. Optical pump-probe as well as fast chemical mixing should be explored as ways to access timescales not available to cryogenic methods.

The group feels that it is essential to define criteria for test specimens needed for studies of radiation damage, sample delivery, and image reconstruction in CDI. A number of different biological samples will be tested with sizes ranging from about 10 nm to 500 nm. A step forward at lower resolution might be to choose nanofabricated objects for the initial phase of the SPI road map towards high resolution. The following characteristics for ultimate test particles/samples are suggested:

- accessible in large quantities (10^{14})
- asymmetric, and of known structure
- composed of many light atoms (i.e., composition of a biological sample)
- in the size range from 10 to 500 nm
- have features with different characteristic lengths that will allow definition of successively higher resolution in reconstructed electron density
- stable in a range of environments for testing alternative delivery methods
- reproducible, ideally with a reproducible internal structure

VI. THE LCLS SINGLE PARTICLE IMAGING ROAD MAP

The working group proposes the following road map to be undertaken in a cooperative fashion involving the global single-particle imaging community as needed and to the extent possible. We propose to systematically develop the techniques of single-particle imaging using potentially non-biological and biological samples chosen for their stability and reproducibility

for XFEL experiments. Continuity of effort over many LCLS experimental runs is expected to be required. We propose that LCLS management allocate necessary beamtime and resources for this purpose and establish guidelines and working principles on how to best engage the community in pursuit of the SPI road map. This road map does not intend to be prescriptive about any technique, sample, instruments of photon energy range to be used along the steps required to achieve the ultimate goal. All such things will be determined during the implementation of the SPI initiative/roadmap.

(1) Characterization of noise profile of the LCLS instruments to be used for SPI. This includes a full characterization of beamline scattering, detector noise measured *in situ*, and evaluation of the harmonic content of the FEL. This is to give an integrated view of the whole system, the reproducibility of the machine on a day-to-day basis and the impact of various electron beam parameters on the scattering properties of the FEL beam.
(2) Background and actual "noise" measurements should be combined with single-particle imaging simulations to determine detection and reconstruction thresholds of the current instrumentation. This will determine whether current background levels are compatible with achieving single-particle imaging, the amount of improvement required to achieve 3 Å resolution, guide sample selection, and possible instrumentation upgrades for proof-of-principle experiments.
(3) A systematic study and improvement of parasitic scatter. This involves, among other things, testing of more apertures and different aperture designs. In addition, the relevant parameter space of photon energy, pulse duration, seeding/bandwidth, and machine parameters need to be optimized for reduced scatter and improved stability. Results will be combined with simulation and the development of "noise tolerant" reconstruction algorithms to determine whether instrument performance is suitable for imaging particles of biologically relevant size ranges (e.g., 30–500 nm objects).
(4) A full characterization of focus profile is required, including power density in main focus, size and shape of beam "wings." This involves a rapid verification of the focal spot size and intensity distribution that can be done at any time with no need to modify the experimental setup. It is critical to be able to verify that the sample is at the best possible focus and that the power density on the sample is maximized and as expected. A pop-in diagnostic that can provide these measurements in a few minutes would be desirable, but ideally another solution providing a non-intrusive shot to shot diagnostic is required. The effect of all upstream optics on focus needs to be characterized, with the possibility to implement rapid feedback tracking to stabilize the KB mirror pointing. An absolute measurement of the photon flux in the focus and jitter is desirable. Absolute focal position feedback needs to be improved, for example, through a laser measuring tool or orthogonal microscope viewing the focal plane.
(5) The structural determination of large objects with low-symmetry are reproducible, or sufficiently understood to $\sim$1 nm with single destructive shots. These objects need not be of biological origin but the challenge of identifying non-biological objects reproducible to 1 nm is recognized. However, previous experience shows how invaluable proof-of-principle demonstrations of known structures, scientifically interesting or not, can be. In the case suitable inorganic objects are used for development purposes, they should contain low Z elements to simulate a biologically relevant molecule. While this specific beamline setup is very different than for single molecules, due to the larger object size and desired resolution, it is deemed useful on the path to develop techniques. Seeking success at a few nanometer resolution will allow for the testing of algorithms and damage models (by varying the pulse length and fluence, assuming there is enough fluence to spare and still get signal). Ideally, proof-of-principle demonstrations at high resolution being difficult enough, such experiments would be decoupled from the equally challenging problem of sample delivery. It is always better to tackle one problem at a time. Should some sample delivery methods known to be unsuitable for single molecules of much smaller sizes be suitable for demonstration experiments with large objects, they should be considered for use. We note that obtaining nanometer-scale 2D projection structures of larger biological objects (cells, organelles) while not in line with the workshop goal of 3 Å structure of biomolecules and not a step directly on the road map, has

some potential for fundamentally interesting biology. Further, achieving nanometer-resolution imaging is an important milestone on the path to 3 Å resolution.

(6) Identify suitable small object(s) to be evaluated and used as test cases for single-molecule imaging. These samples need to be thoroughly characterized for reproducibility and stable structure both before and during sample delivery.

(7) Adequate sample density in focus is a limiting factor in collecting enough data frames for reconstruction to be feasible given a practical amount of beamtime. Various sample delivery methods need to be tested. Sample delivery methods must be fully characterized for, noise, and other effects they might introduce by changing the sample, including sample denaturation and undesired scattering from potential material around the sample.

(8) Select a suitable sample delivery method and best small object to conduct a full single particle imaging experiment.

(9) In parallel with the above experimental developments, it is essential that algorithms be developed, which are capable of dealing with any remaining extraneous effects and sample heterogeneity. Reconstruction algorithms need to be developed that are tolerant to the types of noise and background in a real experiment. Understanding and reducing background is the key element in most successful CDI reconstructions. This requires integration of theory groups into every stage of the experimental road map.

VII. CONCLUSIONS

In response to the proposed road map LCLS has started a Single Particle Imaging Initiative. The goal of the initiative is to solve the technical challenges related to single particle imaging with XFELs and to pave the way for single particle imaging with atomic resolution. In response to a call for participation, 100 scientists from 20 institutions in 8 countries have committed to the initiative. The single particle imaging initiative is allotted special development beamtime by LCLS management and first experiments addressing the road map are under way.

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[29]Meanwhile experiments at LCLS showed high hit-ratios on particles that were clean from contaminants and residues.[5] Further, an analytical solution to the number of required diffraction patterns for a 3D reconstruction has also recently been published.[7] The results show that a 3D data set with 99% probability of full coverage to 3 Å resolution would require on the order of 10^3–10^4 patterns for particles between 10 nm and 70 nm in size. This means that under conditions identical to the experiment by Hantke *et al.*,[5] sample consumption would not exceed 10^7–10^8 particles. Nevertheless, any improvement in efficiency would be beneficial.