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Biomolecular Imaging with Megavolt Electrons: Opportunities and Challenges for an Ultrafast Electron Microscope at SLAC

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Summary

Technology is currently poised at the boundary of three grand challenges in structural biology:

1. High-resolution imaging of isolated biomolecules of varying sizes, enabling the determination of atomic models.
2. Tomography at sub-nanometer resolution with a wide field of view, showing the structural arrangement and positive identification of chemical components in a cellular environment.
3. Biomolecular dynamics at nm-μs resolutions, enabling visualization of biomolecular function: complex assembly, signal transduction, folding, *etc*.

Recent developments in cryoEM instruments (direct detectors) and x-ray sources (XFELs) have brought these goals tantalizingly close. One promising avenue to addressing all three goals is to advance the electron source technology behind electron microscopy, improving the performance of electron microscopes to encompass one or more of the above goals.

In this paper, we consider the construction of a pulsed MeV instrument to further these goals, and conclude:

1. Single-shot (including diffract-before-destroy) imaging modes of atomic structure are likely impossible with electrons. Current technology is very far from making this achievable, and the requirements are close to the limits dictated by quantum mechanics for relativistic electron beams.
2. It is not clear that MeV beams provide a clear advantage to image formation via improved damage, detectors, sample thickness, or jitter removal.
3. MeV beams can provide a modest improvement to resolution via improved contrast transfer
4. An emphasis should be placed on increasing the average (vs peak) brightness of electron sources, enabling increased coherence and decreased image formation time. Radio-frequency (RF) technology producing pulsed MeV beams is one possible route.

We conclude that pulsed MeV beams are expected to provide modest advantages for biological imaging. Such beams, based on *e.g.* RF accelerator technology, may be more strongly motivated by applications in other fields. This paper should provide a framework for evaluating motivations provided by other fields as well.

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I. Scientific Motivation

Recently, cryogenic electron microscopy (cryoEM) has enjoyed an explosion of activity in the field of structural biology. Spurred by the advent of direct-electron detectors, the ability for cryoEM to image aperiodic biological samples on near-atomic scales has significantly advanced. CryoEM can now elucidate structures of large biocomplexes that were previously intractable to competing techniques, most prominently crystallography. Such structures greatly advance our ability to understand and re-engineer biological components such as proteins for medical, energy production, and materials purposes. Their importance is difficult to overstate. Thus, a number of institutions, including SLAC and Stanford, have begun to invest heavily in a number of state of the art electron microscopes for cryoEM studies of biological matter.

CryoEM is still limited, however, to large biological complexes (typically >150 kDa, record 65 kDa [1]) and moderate resolutions (>3 Å). If the technique could be routinely applied to determine the structures of smaller specimens (<100 kDa), the number of possible targets for structure determination would increase dramatically. Further, if the resolution could be improved for such targets, to <2 Å, it would enable the unambiguous placement all non-hydrogen atoms in the structure. Improvement in cryoEM technology is necessary to bring structural determination of nearly all biomolecular structures into range.

A similar precipice is seen in cryogenic electron tomography (cryoET), where an improvement in resolution by a factor of sub-nanometer resolution enables positive identification of protein species and complexes in place in their cellular environment. Such information would be invaluable in *e.g.* understanding neurotransmitter function.

Finally, the direct measurement of biomolecular dynamics at nm-μs resolutions is a dream of biophysics, that could directly address how molecular signaling occurs (for instance upon drug binding), how proteins fold, and how enzymes (biological catalysts) function. The field of atomic-resolution molecular simulation has invested billions of dollars in computers to simulate such processes; the impact of an instrument that could directly measure them, without the worries incurred by the accuracy of a model, is hard to overstate.

Current microscopes still underperform in achievable resolution compared to their theoretical limit. Between current capabilities and this limit are a large number of important scientific targets. What engineering developments can advance EM technology to include those scientific targets? Novel electron sources are one possibility. Here, pulsed MeV sources, of the kind commonly found in particle accelerators, are assessed for their ability to improve the resolution of static imaging (microscopy or tomography), where the samples are presumably under cryogenic conditions.

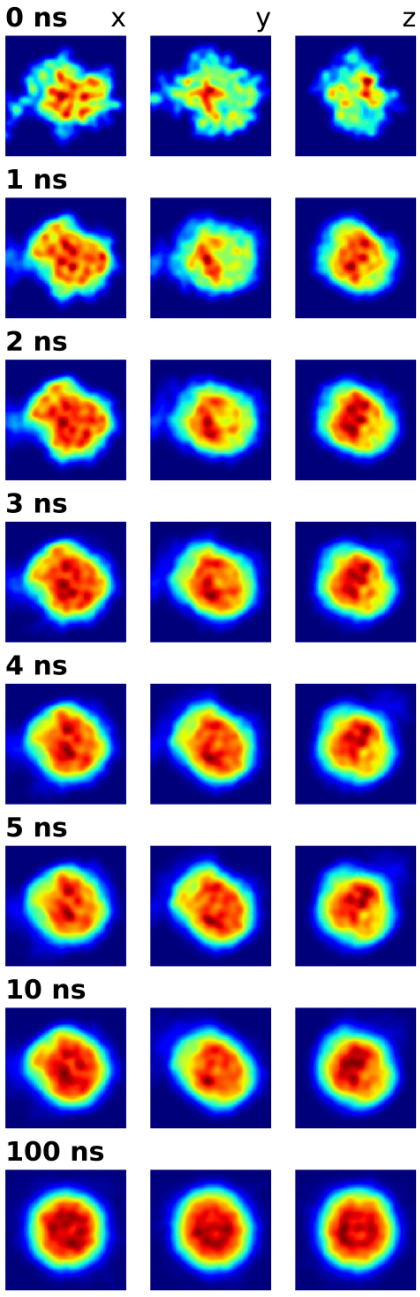

Figure 1. Protein tumbling requires 1 ns image acquisition time. Shown are simulated images of the protein ubiquitin expected from an "ideal" electron microscope acquired over the interval indicated ("0 ns" corresponds to instantaneous acquisition). As the protein molecule undergoes global rotation due to diffusion, images are blurred and information is lost. After a few ns, atomic information is lost; after 100 ns the image is nearly isotropic. To generate images, a molecular dynamics simulation of ubiquitin was performed in explicit water at 300 K (simulations generously provided by DESRES [2]). For each integration time shown, the atomic structure was extracted from 50 equally spaced intervals across that time. The 3D electron density map was computed from the Cromer-Mann model onto a 30^3 0.25 Å grid and projected onto a cardinal axis, indicated.

Also considered is the possibility for electron microscopes to perform meaningful dynamical measurements. Any dynamical measurement of biological matter must give up the significant advantages of cryogenic conditions, which helps limit radiation damage and fixes the specimen in place during measurement. Indeed this last point is significant: while most biologically significant processes occur on μs or longer timescales, because of molecular tumbling, micrographs must be developed in <1 ns to avoid blurring due to unwanted global rotations and translations (Figure 1). This sets a hard limit on the spatiotemporal resolution such an instrument must achieve to be routinely useful: 1 nm-1 ns image formation (5 e- / $Å^2$ dose) would be required.

This poses a serious – perhaps insurmountable – challenge for electron imaging. As discussed in Section III, beam brightness requirements place this spatiotemporal resolution well outside the limits of what is currently possible, and close to the fundamental physical limit. Thus, before pursuing such imaging, one of two tactics must be demonstrated to deal with molecular tumbling. First, the sample can be physically anchored. This is intellectually straightforward, but may be difficult in practice. Second, the image acquisition split into many sub-ns exposures and corrected for molecular tumbling in computational post-processing. This second tactic would be supported by a high rep-rate pulsed instrument (Section III) but would require a significant advance in detector readout speed and computational capabilities that seem distant. Neither of these tactics is well studied or established, and must be investigated if dynamical biological imaging is a primary instrumentation goal.

II. Historical Instruments: MeV and Dynamic EM Instruments

The first electron microscope (EM) was demonstrated in 1933 by Ernst Ruska, for which he was awarded the 1986 Nobel prize. Since then, three major technological developments have resulted in significant advances in the capabilities of EMs: first, the introduction of field emission sources, then the advent of spherical aberration correctors, and finally the use of fast, high quantum efficiency direct electron detectors.

While EMs have imaged inorganic matter to 10s of pm resolution, clearly showing individual atoms, results on soft matter such as proteins and other biomolecules have consistently underperformed when compared to theoretical limits. Atomic resolution is rarely reached for biological samples, and then only through averaging many independent images. The optical system of modern EMs is in principle limited by chromatic aberration, but given the resolution discrepancy between hard and soft matter, it seems unlikely that biological imaging is limited by chromatic aberration arising from the objective lens. Practical limitations outside of the scope of the imaging system, for example beam-induced motion, alignment, sample-induced aberrations, must be considered and mitigated.

This paper explores the possibility that the next revolutionary leap in EM technology will come from high-brightness MeV electron sources. Such a source could open possibilities for dynamic imaging, but also enable current limits on static image formation of soft matter to be lifted. Both MeV and keV “dynamic” TEMs have been constructed in the past, and those efforts are briefly reviewed here.

1. High Voltage Instruments

Megavolt electron microscopes were constructed in the 1970s [3], but these instruments were typically in-house projects that were not commercialized. Today the vast majority of microscopes sold accelerate electrons to 80-300 keV. It is prudent to understand why construction of megavolt instruments fell out of favor and the risks associated with pursuing this path.

Historically, a significant motivation for pursuing MeV instruments was the hope that biological live specimens could be studied. Unfortunately, it was immediately clear that this would not be possible, with live cells quickly dying under exposure to the intense electron beams necessary to form images [3]. A second hope, which was borne out, was that MeV energies would enable study of significantly thicker sections than keV energies. Indeed, clearer images of biological structures, for example, were witnessed with high accelerating voltages [3].

Despite this advantage, a major reason to limit accelerating voltages to 80-300 keV is cost. Accelerating structures and associated power sources for MeV instruments require significant investment. MeV instruments constructed have been >100 tons in weight and taken up considerable space (*e.g.* [4]). Further, radiation concerns due to MeV electrons require expensive shielding schemes and powerful lenses, which are typically large (order tons). Therefore, there must be a clear and significant advantage to such instruments to justify the cost, which was not the case. It is prudent to remember that a modern attempt will also be expensive, and pursuing the ideas below will come with a price tag.

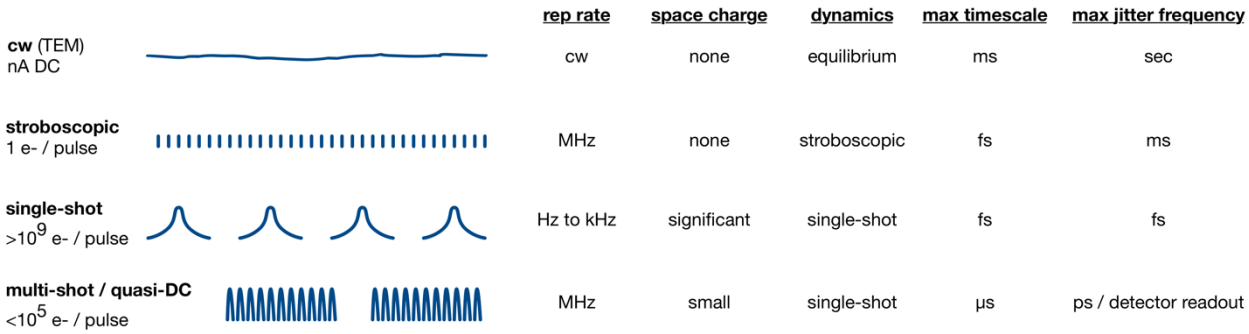

Figure 2. Schematic of electron pulse delivery schemes, along with their advantageous and disadvantageous properties.

2. Time-resolved Instruments

One of the most exciting potential capabilities of a next-generation electron microscopy instrument would be the ability to collect time-resolved images. All biological components move to function, whether producing or consuming energy, transmitting signals, performing catalysis, or reproducing. The essential processes of life are dynamic.

One can consider three possible modes of operation for a time-resolved instrument (Figure 2):

- In an *equilibrium* mode, a microscope would form an initial image at “time zero” followed by another image at some “delta-t”. Repeated measurements could be combined to form a comprehensive view of the dynamics of the system [5]. Such operation requires measuring the same (single) molecular entity twice, placing strict demands on the capabilities of the microscope.
- In a *pump-probe* mode, the system would be placed in a (possibly irreversible) non-equilibrium state by e.g. rapid mixing or irradiation with an optical laser at time zero, and the subsequent time evolution of a single molecule or ensemble followed by forming one or more images.
- Finally, in a *stroboscopic* mode, the system would undergo a non-equilibrium cycle many times. During each cycle, at least one measurement would be made, and these measurements could be combined to form an image. This mode places the least severe requirements on the microscope, as the measurement may be repeated and averaged, but is only applicable to samples that can be exactly reproduced in their original state.

Electron imaging instruments operating in each of these regimes have been constructed previously.

Modern TEMs such as the FEI Titan may be equipped with environmental cells providing a hydrated sample environment can be considered an example of the equilibrium class of instruments. While such instruments are suitable for study of non-biological matter, they acquire images too slowly (ms-secs) to be useful for biological imaging. Further, the quality of imaged obtained from samples in environmental cells tends to be compromised, limited to ~10s of nm [A. Koh, personal communication].

When a sample can be consistently prepared in a reproducible state, it is possible to take many measurements at a dose insufficient for a single image, and average them to produce the final interpretable image. This tactic, known as stroboscopic imaging, has been employed by, for example, the UED facilities at Caltech, SLAC, and elsewhere [6,7]. Unfortunately, few (if any) biological objects can be readily prepared in exactly the same orientation and excited state necessary for stroboscopic imaging. The number of samples that could be studied in this fashion is too small to justify an expensive instrumentation project.

Finally, and perhaps most promisingly for dynamical imaging of biological matter, is a single-shot instrument. The DTEM project is the most prominent example of such a microscope (see section VII.3). Delivering electrons in a bunch reduces the theoretical performance of the microscope due to space-charge effects – as they travel down the microscope column, the bunch will spread and change path due to internal Coulomb repulsion, and *not* strictly due to interaction with the sample or microscope optics. A continuous source avoids this by only having (on average) a single electron in the column at a time. Thus, pulsed instruments incur an intrinsic design penalty, and must take care to minimize the impact of space charge on image quality.

In fact, this space charge limitation places the nm-ns spatiotemporal resolution desired to form atomic-scale images in less than the molecular tumbling time of proteins (Figure 1) very close to the theoretically possible limit dictated by the Pauli exclusion principle (Figure 4). As previously discussed, imaging protein motions at the atomic scale will therefore require either physical fixation or rapid image readout combined with techniques to reconstruct a blurred image from many incomplete (low-illumination) exposures.

3. Historical Outlook

It is worth understanding that a pulsed instrument and megavolt accelerating energies go hand in hand. Accelerating the electrons to relativistic energies greatly reduces space charge concerns (transverse space charge forces scale as $1/\gamma^3$, with γ being the Lorentz factor). At the same time, accelerating schemes for producing a bunch of MeV electrons are well understood in the particle accelerator community. Thus, it is rational to advance these two concepts together when pursuing the next generation of electron microscopes.

In this paper, the view is taken that it may be worth re-visiting the electron source as a primary route to advancing the capabilities of electron microscopes to image soft matter, in both a static and dynamic capacity. While historical efforts to produce MeV instruments or pulsed instruments have not gained wide acceptance, recent technological advances in electron source

technology – especially in the development of superconducting sources constructed for XFELS – mean it is time to revisit the scientific and technical opportunities afforded by these designs, *especially* when combined into a pulsed-MeV instrument. The possible advantages of such an instrument are described below.

III. Design Considerations for a High-Current MeV Source

There is an intrinsic tradeoff between temporal resolution and spatial resolution in electron imaging systems. It takes a certain number of electrons (per unit area) to generate a complete image, and this number increases with spatial resolution. To increase temporal resolution, this total number of electrons must be delivered more rapidly.

In a pulsed instrument, electrons are delivered in a bunch – if the image can be formed by a single bunch, it is the *peak* current that dictates the temporal resolution, and requirements on the *average* current can be relaxed. Previous electron microscopy instruments have therefore selected a target timescale (including static imaging as a special case) and then stretched an image-forming electron dose as broadly as possible over that timescale. The DTEM, for instance, follows this design principle (VII.3) [8], where a single pulse of 10^9 electrons is used to form a nm-resolution image in a single shot in order to achieve 10s of ns temporal resolution. Continuous sources can be considered a special case of a pulsed instrument with infinitely long pulses. This has led to the continuous sources used in TEMs, where and the current is set to resolve images in a reasonable amount of time (< second).

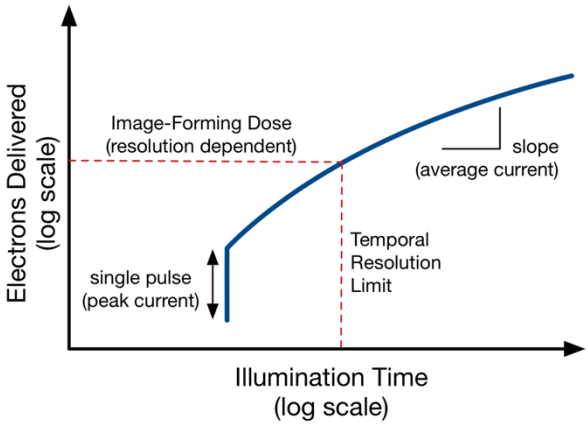

Figure 3. Temporal resolution is a function of beam current. Image formation at a specified resolution requires sufficient areal dose to the sample. If this is achieved in a single pulse (single-shot mode), then the pulse duration sets the temporal resolution of the instrument. Otherwise, many pulses must be averaged, and the average current dictates the temporal resolution.

An increased peak current, however, implies that electrons are spatially localized, and therefore subject to space-charge broadening. This places a hard limit on spatial resolution. This point should be emphasized: **space charge forces in electron imaging place a hard limit on spatiotemporal resolution** (Figure 4). The only way to extend this frontier is to move to higher accelerating voltage, where relativistic broadening mitigates space-charge forces, though this tactic is practically limited to ~10 MeV, after which the electrons are energetic enough to perform nuclear reactions, causing large amounts of damage [9] and secondary radiation.

This paper considers a quasi-DC pulsed instrument capable of operating flexibly near the space-charge limited frontier of spatiotemporal resolution. This could be accomplished with a

high-rep rate (*e.g.* 100 MHz) operation delivering <100 ps pulses of ~10^7 electrons per bunch (peak current: 100 mA, average current: 100 µA). Such an instrument would be capable of delivering order 1 e- / Å^2 to a 10 µm spot at a 10 µs timescale for biological imaging. This design would enable a tradeoff of temporal-spatial resolution along the 10^{-15} m-s frontier, from 1 Å / 10 µs to 100 ns / 10 nm to 100 ps / 10 µm. While the "slowest" mode would be most useful for biological samples, faster operating conditions would have significant impact in other fields, as outlined in the 2014 DoE BES report on the Future of Electron Scattering and Diffraction [10].

Key to this instrument is maintaining high current (and therefore spatiotemporal resolution) by operating at a high rep rate. While such an instrument cannot match the theoretical spatiotemporal performance of instrument designed for a single timescale (with pulse length to match), in return, gains the opportunity to:

1. **operate flexibly**, studying static or slow timescales with spatial resolution comparable to a cw source; stroboscopic phenomena with performance nearly identical to dedicated stroboscopic instruments; and performing single-pulse like measurements from small trains of pulses (though at lower temporal resolution)
2. **minimize the effects of system jitter** on image formation by reading out the system camera after every pulse or every few pulses and performing in-software image alignment to reconstruct a jitter-free image
3. **leverage existing high-field low-emittance electron sources** typically used in particle accelerators, especially rapidly developing superconducting linac sources being commissioned for XFELs

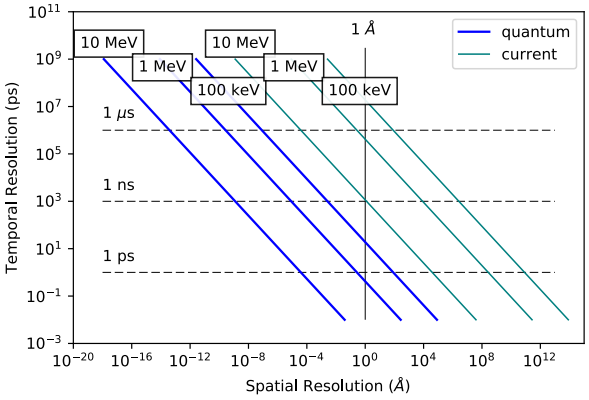

Figure 4. Space-charge effects limit maximum attainable spatiotemporal resolution. Lines show achievable spatiotemporal performance given the beam brightness limits dictated by quantum mechanical theory (blue lines) and current technology (9 decades worse, teal lines). Increasing the accelerating voltage of the electrons used for imaging increases the maximum attainable spatiotemporal resolution. The 1 Å line indicates the useful limit for molecular imaging. Single-shot diffract-before-destroy imaging is expected to require pulses <1ps. Assumptions: 5 e- / Å^2 to form image, 10^{-4} energy spread, beam divergence is 100x objective acceptance angle, 1 mm C_s, spherical aberration is resolution limiting. Based on equations 4 & 10 from Callaham with small angle approximation (Callaham 1988. IEEE J. Quant. Elec. (2) 1958-1962).

MeV operating energies carry additional benefits, for both time-resolved and static imaging:

1. **improving contrast transfer** for weak-phase objects and ensuring the projection approximation holds at high resolution
2. enabling study of more than a few atomic layers **without chromatic aberration** incurred by multiple scattering in the sample, necessary for hydrated (and perhaps frozen) biological samples
3. **mitigating space-charge** effects in high-brightness beams

Details of each of these advantages are discussed in Section VI. Further, the possibility for such an instrument to match or exceed current microscope performance in all other relevant categories is evaluated.

IV. Diffract-before-destroy Imaging with Electrons

Electron microscopes are limited by the quantity of structural information obtained for a given amount of radiation damage inevitably incurred by the electron beam. Achieving the scientific goals stated in Section I comes down to maximizing the information-to-damage ratio.

Worth considering, therefore, is the possibility of operating in a diffract-before-destroy mode similar to modern XFEL sources. In this scheme, a single, short (sub-ps duration) pulse of high intensity radiation is employed for imaging. The sample is destroyed, but not before the radiation diffracts from the sample, capturing unperturbed information. In this scheme, the radiation dose does not limit the imparted information; only the intensity of the pulse does, making it appealing for imaging of radiation-sensitive samples such as proteins. This technique has been explored extensively at LCLS in relation to the imaging of aperiodic biological matter via the Single-Particle Imaging (SPI) Initiative [11]. The SPI initiative has solved structures at 9 nm resolution [A. Aquila, personal communication] to date. While SPI promises opportunities, especially in the study of dynamics, beyond static structures, it is worth noting this resolution is substantially worse than the ~3 Å structures being produced by state of the art cryoEM instruments.

In 2015, Ray Egerton analyzed the possibility of performing a similar scheme with electrons from a theoretical point of view [12]. Unlike x-rays, electrons interact with one another, and repulsive space-charge effects make it difficult to produce the high-intensity, short duration, spatially coherent pulses necessary for diffract-before-destroy imaging. These issues are mitigated by operating at MeV energies, where relativistic effects reduce space-charge forces. Nonetheless, Egerton concluded that even at MeV energies, diffract-before-destroy imaging with electrons for nm-scale aperiodic objects could not match the capabilities of XFELs.

An experimental analysis corroborates this: we can compare the current performance of existing electron diffraction instruments to the LCLS.

First, assume that diffract-before-destroy imaging with electrons and x-rays is directly analogous. This may not be the case, but seems the best approximation currently available given the state of ultrafast damage studies with electrons. While the mechanistic details of diffract-before-destroy imaging at LCLS are still a topic of intense study, it is clear that most useful information is imparted within at least the first 150 fs of the x-ray pulse [K. Nass, presentation given at PSI Sept. 2014], and possibly within just a few fs [11]. Here, we conservatively assume that *some* useful information will be imparted by a destructive electron pulse over 1 ps, and that after that point the sample's electronic structure is sufficiently disrupted so as to be considered "destroyed".

The LCLS generates ~10^{12} photons/pulse in a fully coherent beam. To image a ~100 nm virus at 10 nm resolution, this beam is typically focused to ~1 μm. To match the coherent imaging capabilities of the LCLS, an electron source with:

- 10^7+ electrons (2 pC) per pulse
- 100+ nm coherence (both transverse and longitudinally)
- 0.15 $Å^{-1}$ q-resolution (100 nm imaging region, 10 nm voxel size, 2x oversampling)
- 1 μm spot size
- < 1 ps pulse length

would be required. We have assumed the electrons interact 10^5 times as strongly as the x-ray beam, a generous estimate for the soft x-rays employed for SPI at LCLS. These beam parameters imply a source producing 2 pC pulses with 0.1 nm-rad transverse emittance at the sample. For comparison, the SLAC UED produces pulses of 10^5 electrons with 3 nm-rad emittance. Thus, the SLAC UED source would require a factor of 30x improvement in emittance to match the LCLS [R. Li, personal communication]. Considering the LCLS RF injector used at SLAC's UED facility is already a state of the art gun [7], It is not currently clear how to pursue such an advance, which if possible, would require a revolution in electron source technology.

Further, increasing the source brightness and focus, necessary to improve the technique from 100 nm objects to more desirable Å-scale images, will result in a dramatic increase in space charge forces that will blur any resulting image. Increasing the resolution of an image by a factor of 2 in each dimension necessitates a 4x increase in total dose. A resolution improvement from 100 nm to 1 nm would require at least a 10^4 increase in electron dose, an extremely bright 20 nC, 1 ps pulse. Therefore, at this time, it seems prudent to set aside the hopes of diffract-before-destroy imaging with electrons.

V. Coherent Imaging and The Necessity of a Microscopy

Considering the success of ultrafast electron diffraction (UED) instruments operating at MeV energies, the increased cost and complexity of a microscope must offer a concomitant advantage over a diffraction instrument. The major theoretical difference between the two modes is the use of a lens in microscopy to retrieve the phase of diffracted light, directly producing an image. Diffraction instruments rely on inference to determine these phases.

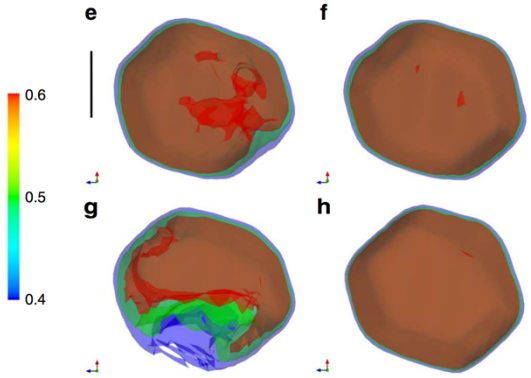

Figure 5. The effect of partial coherence on coherent imaging. Coherent diffractive images were taken of a gold nanoparticle employing partial coherence (left, e and g). When the lack of coherence is accounted for, defects in the images are removed (right, f, h) [13].

Coherent imaging and ptychography, which rely on phase retrieval algorithms, are continually improving, proving that real-space information can be readily extracted from diffraction images [14]. Current UED instruments, however, lack the coherence necessary to perform coherent imaging. This limits the resolution achievable for aperiodic samples. Coherent imaging with electrons has, however, been effectively applied to periodic (*i.e.* coherent) samples with success [15,16]. Further, work on performing coherent imaging with partially coherent sources has progressed [13], possibly alleviating the necessity for a fully coherent source. Nonetheless, it is highly desirable to design systems that do not rely on reconstruction schemes to overcome shortcomings in the optical setup, and coherent imaging for high resolution applications should only be considered if a sufficiently coherent source (100+ nm in both transverse/longitudinal coordinates) can be produced. Presumably spatial coherence can be achieved through alignment and apertures; temporal coherence is more demanding, placing requirements on the electron source or external monochromators.

Given that an electron microscope can always be operated in diffraction mode, a recommendation is made: if at all possible, any MeV microscopy project should *first* consist of a diffraction-only phase, where the possibility of a ptychographic instrument is investigated, before proceeding to install an objective lens to finish the microscope.

One final possibility to consider is a hybrid instrument. Such an instrument would be equipped with an objective lens, but it would only be used to acquire low-resolution real space images. These images would then be used as priors for phase retrieval from diffraction images taken from the same instrument. This has the advantage of eliminating the objective lens as a source of potential aberration, while ensuring the diffraction image phases can be robustly determined. Such schemes have been recently investigated [17]. A drawback to this approach it that it requires two images to be formed, increasing the radiation damage necessary to acquire useful information.

VI. Addressing the Limitations of Static Imaging via an MeV/Pulsed Design

Current instruments operate well below the theoretical resolution limits. Glaeser and Hall have recently reviewed the current limitations of EM performance [18,19], identifying four technical factors that may explain this gap: imperfect contrast transfer from the microscope optics, mechanical jitter in the system, imperfect detectors, and multiple scattering due to sample thickness. Finally we discuss perturbations due to external magnetic fields, which while unlikely to limit the resolution of a first instrument, have been the source of resolution limits in other microscopes and would be mitigated by MeV beam energies. Also considered is the impact electron energy has on the number of elastic scattering events, which impart useful information, to inelastic scattering, which incurs radiation damage on the sample.

One final note: while not strictly a function of a pulsed/MeV design, higher source current than is currently employed in electron microscopes may significantly aid image formation. Such sources can be delivered via strict apertures and monochromators to deliver a highly coherent beam that

is still bright enough for image formation. Such a beam would be less sensitive to optical aberration. This simple fact could enough to justify building a new electron source for static imaging.

1. Contrast Transfer

Because biological samples are light and thin, nearly all the contrast in EM images of biological samples is due to phase contrast. Current electron microscopes enhance this contrast by operating with underfocus, and compensating with spherical aberration which can be controlled by advanced correctors [20,21]. This setup limits the maximum information that can be imparted, and results in a high-contrast but (relatively speaking) blurry image.

The ability of a microscope to faithfully reproduce an image of the illuminated sample is often assessed by the instruments contrast transfer function (CTF), which represents how the microscope's optical system filters spatial frequencies in the formed image. A large, flat passband results in high-resolution images with little delocalization error. For the well-studied weak-phase case, and at fixed aberration and defocus values, decreasing the wavelength of the electrons used for imaging is *universally* predicted to increase the image quality by extending the point resolution (first zero in the CTF) and delocalization (Figure 6). For very high-resolution imaging, MeV energies further aids in image interpretability by ensuring that images correspond to projections of the sample's electric potential. The flat Ewald sphere provided by high energy electrons contrast results in a distortion free projection of the sample's structure high resolution [22].

Microscope performance could be enhanced (at any operating energy) using in-focus imaging with a phase plate, an optic that provides direct phase contrast. While development of a satisfactory phase plate is still underway, recent advances are promising [23] and have yielded significantly improved images for biological specimens [24,25]. MeV energies improve contrast transfer in phase plate systems in the same manner as out-of-focus phase contrast.

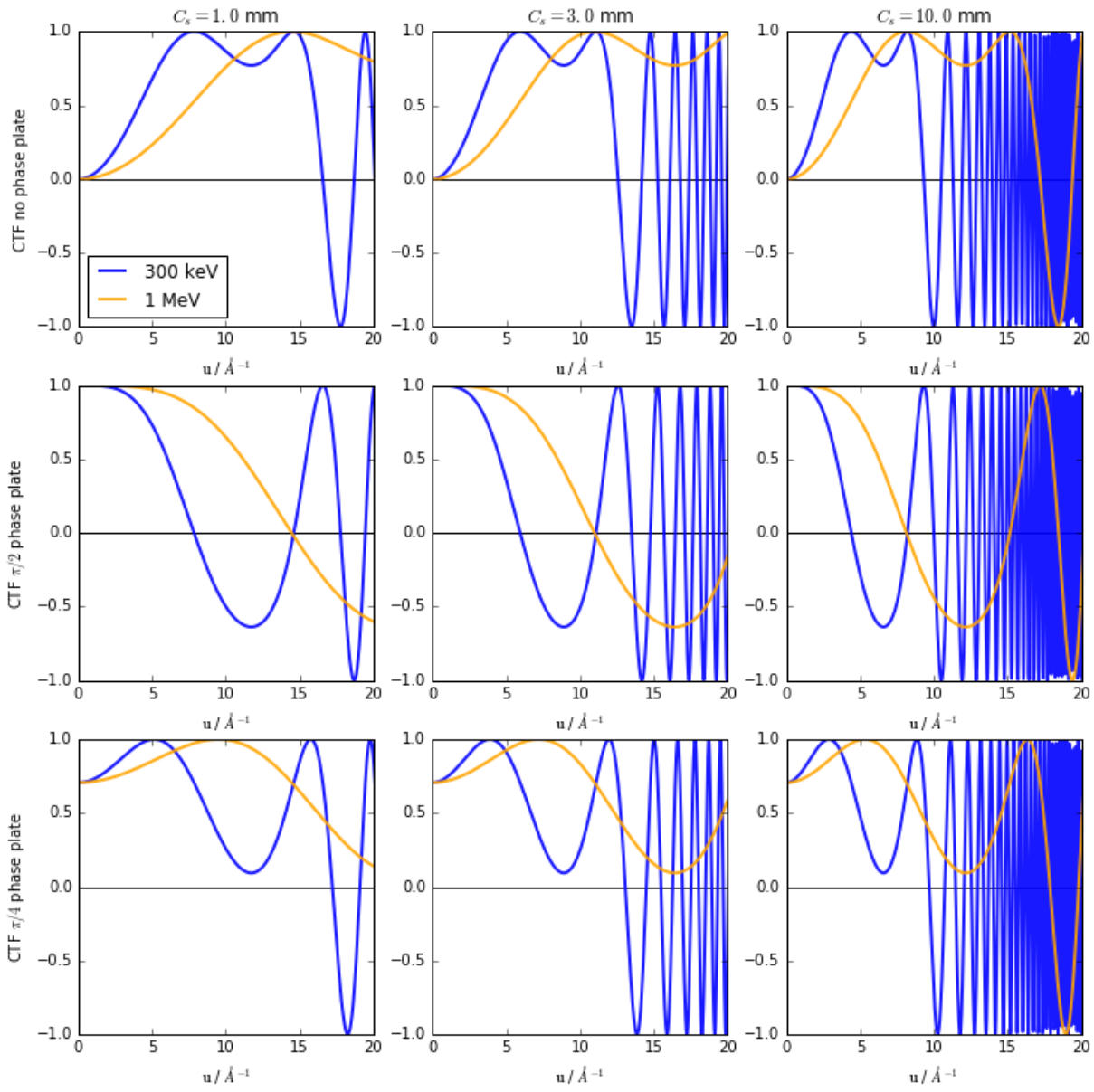

Figure 6. Predicted contrast transfer functions (CTFs) show the benefits of high accelerating voltages and wave plates. Shown is the predicted ideal weak-phase object approximation CTF under various accelerating voltages (blue 300 keV, orange 1 MeV), spherical aberration coefficients (C_s, columns), and phase plates (rows). All microscopes are assumed to operate at Scherzer defocus [26]. Only defocus and spherical aberration are considered, no other envelope functions or aberrations are included, and C_s is fixed. Increasing accelerating voltage extends the useful passband for all conditions. Note that the passband of the phase-plate shifted CTFs can be further "improved" if simultaneous adjustment of the phase shift, defocus, and spherical aberration (via a corrector) is considered.

2. Sample Jitter

In modern electron microscopes, images are acquired over ms to seconds timescales, and all microscopes suffer from sample motion that occurs during this time. The two primary sources of motion are sample stage drift and beam-induced motion. Ultra-stable stages and sample grids

have demonstrated sample jitter is a limiting factor in image quality [27,28]. When imaging at Ångström scales, the tolerance for sample motion is correspondingly tight. Such drift may be partially addressed by image alignment using modern fast readout cameras [28] and alignment algorithms. Beyond mechanical components, sample charging due to the electron beam itself is significant and difficult to overcome [27].

Computational alignment offers the possibility of eliminating mechanical jitter on timescales longer than the exposure time required to form an image with enough information to be aligned unambiguously. If an image can be formed in a single pulse, the pulse length sets this timescale; otherwise, the average current of the microscope sets this timescale.

Such a scheme relies heavily on computational capabilities, which must align images that will be potentially sparse. The fewer electrons needed to align images, the more powerful the technique. Global alignment algorithms have been developed (*e.g.* [29]). Still, the specific application of such algorithms to very sparse (low-light) EM data remains an unproven technique, and the limits of such approaches are an open research question that should be pursued.

While seemingly mundane, the issue of mechanical noise and beam deflection due to charging are likely major limiting factors in the performance of EMs operating at Ångström resolutions.

3. Sample Thickness: Minimizing Chromatic Aberration

Samples for electron microscopes must be less than the mean free path of the electrons used for imaging to avoid chromatic aberration caused by multiple scattering in the sample. For 300 keV instruments, this means samples must be thinner than a few 100s of nanometers. The mean free path of 1 MeV electrons in carbon is 5x longer than at 300 keV [30], expanding the acceptable thickness of samples proportionately.

Multiple scattering in low-voltage TEMs causes chromatic aberration that cannot be addressed through objective lens design or correctors. This aberration blurs the resulting micrograph, limiting resolution. The problem is especially important in tomography [31] and for hydrated “environmental” samples. In tomography, it is challenging to microtome 100s nm scale sections and use of thicker sections aids sample preparation and data analysis. In hydrated/environmental imaging, necessary for any dynamical studies, forming ~100 nm thick flow cells is nearly impossible, as the thin membranes used (typically 50 nm SiN) bow outward when loaded with sample, resulting in chromatic aberration at 300 keV [A. Koh, personal communication]. Thus, high energy electrons will provide a massive practical advantage, and perhaps be necessary, for dynamical microscopy studies of biomolecules in their native, hydrated state.

4. Detectors

The recent development of direct electron detectors with fast readout rates and high quantum efficiencies has recently revolutionized biological imaging with cryoEMs [32]. Of these cameras,

the most notable is the Gatan K2, the commercial version of the TEAM camera developed at LBNL [33].

Recently, an MeV-compatible TEAM sensor comprised of 1k by 1k, 9.5 μm pixels has been commissioned at the SLAC UED source. This camera is very similar to the original TEAM design, but has a 3 times thicker sensor designed to capture penetrating MeV electrons. The camera can read out at 500 Hz and has demonstrated electron counting capabilities, though requiring some data processing [T. Vecchione, personal communication]. This success seems to indicate that while some details may change, cameras for MeV EMs comparable to those in use for keV instruments should be possible to fabricate. Further, because MeV electrons carry significantly more energy per particle, it seems likely that with development MeV cameras may exhibit higher quantum efficiencies and better signal to noise than their low energy counterparts.

It should be noted that faster cameras are under development and may be very advantageous for the kind of pulsed instrument discussed in this paper. Currently, the LBNL group is working on a 576^2 pixel camera that can read out at 100 kHz [P. Denes, personal communication]. Operating at high readout speeds matched to a high pulse rate will maximize the advantages of a pulsed instrument discussed here, especially if electron counting performance can be maintained.

5. Sample Damage

Reports in the literature concerning the effect of different accelerating energies on sample damage in TEM instruments present conflicting views. Based on the ratio of predicted elastic and inelastic cross sections for carbon, Henderson suggested that between 100 keV and a few MeV, the information-to-damage ratio should not change much [9]. In the early 1970s, radiation damage studies of bio-matter followed the construction of the first high voltage TEMs (1-3 MeV). There was significant hope these instruments would enable imaging of live biological specimens. Dupouy reported that “from experiments carried out to 1 MV, it can be seen that radiation damage is, as far as valine is concerned, at least three times less at 1 MV than at 80 kV” [4] based on the work of Thomas and Lacaze [34]. Finally, Cosslett has summarized reports that while electron radiation still quickly killed specimens, fungal spores survived longer at higher voltage for a given dose [3].

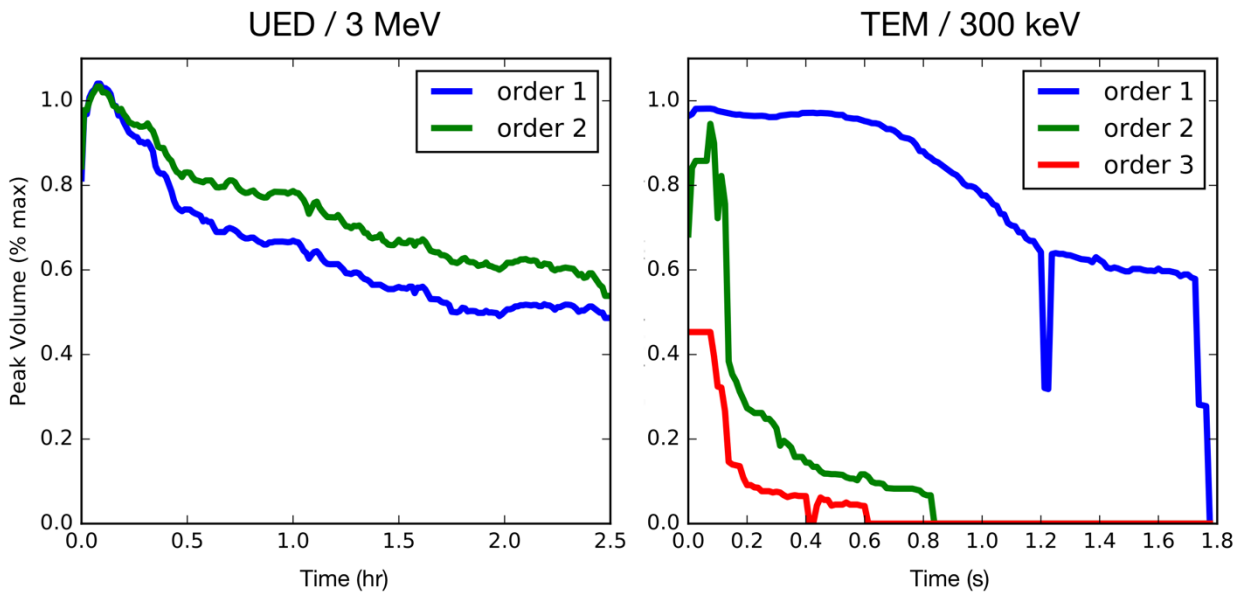

Figure 7. Damage studies at the MeV SLAC UED demonstrate a different damage mechanism than at a commercial TEM. Single crystals of paraffin grown on carbon/Cu100 TEM grids were exposed to the beams from these instruments and diffraction patterns recorded. Damage was monitored as Bragg reflections of different orders (scattering angles) diminished over time. Rate of damage can be quantified by the critical dose, at which an integrated peak reaches 1/e of the original intensity. For the MeV data, obtained at a flux of $9x10^{-5}$ e A^{-2} s^{-1} successive orders exhibit 0.40 and 0.46 e A^{-2} critical doses. At 300 keV and a flux of 0.54 e A^{-2} s^{-1} critical doses of 3.13, 1.19, and 0.97 e A^{-2} were measured.

To address this situation, damage studies were conducted at SLAC using both the MeV UED source and a commercial TEM (FEI Titan) operating at 300 keV. Single crystals of paraffin grown on carbon/Cu100 TEM grids were exposed to the beams from these instruments and diffraction patterns recorded. Damage was monitored as Bragg reflections of different orders (scattering angles) diminished over time (Figure 7).

Unfortunately, the damage mechanisms between the two sets of data (UED and TEM) appear qualitatively different. Bragg peaks in the TEM data decay in a biphasic fashion, with high-order reflections definitively disappearing at a faster rate. In the UED, the decay rate is more or less uniform for all Bragg peaks and occurs with a single timescale. The rates of decay are of the same order of magnitude when normalized for electron flux per unit area. These sources are extremely different: the UED is a low current machine ($5x10^{-9}$ e-/Å^2-s *vs.* 0.54 e-/Å^2-s in the TEM) that operates with 100 fs pulses delivered at 180 Hz (*vs*. CW for the TEM). Dose-rate effects have been witnessed in TEMs for conditions that are much more similar [35].

These differences lead us to conclude that the damage mechanisms are likely different between the two instruments, and we cannot draw firm conclusions about the effect of accelerating voltage alone. It is unlikely, however, that the difference in damage rate between the two sources by more than an order of magnitude.

While much work has clearly been done to study the effects of radiation damage as a function of accelerating voltage, it is striking that none of the studies reviewed here can provide a

quantitative analysis. It is currently unclear if MeV energies will offer a benefit for biological imaging by limiting radiation damage for a given quantity of information imparted. At the same time, it seems that any difference in this regard will be minor, and is likely not to be worse at MeV than keV energies.

6. Mitigating External Magnetic Fields

Recently, it was reported that very small (order 0.1 nT) magnetic fields due to Johnson noise in microscope components limited the performance of a state of the art microscope. Thermal magnetic noise caused beam deviations that limited the sub-Ångström resolution of an aberration-corrected instrument operating at 80 keV [36]. Johnson noise was pinpointed as the root cause by cooling the electron flight tube of the microscope; corresponding enhancements in beam coherence were seen. While unlikely to be the cause of limited resolution for biological specimens, operating microscopes at high accelerating voltages will mitigate the effects of any unshielded external fields, as the electrons in the beam carry more momentum.

TABLE 1. STANDARD OPERATING PARAMETERS

	Titan TEM	SLAC UED	Madison Gun	DTEM	Proposed UEM
E- ENERGY	80/200/300 keV	2-5 MeV	4 MeV	200 keV	1 MeV
PULSE DUR	cw	100 fs	70 fs	10-1000 ns	100 ps
# E-/PULSE	1	10^5	10^9	10^9	10^7
REP RATE	cw	180 Hz	5 MHz	10 Hz	100 MHz
AVG CURRENT	nA	3 pA	1 mA	nA	100 μA
PK CURRENT	nA	100 mA	>1000 A	10 mA	100 mA
DIV AT SMPL*	parallel	200 μrad	100 mrad**	parallel	< 100 μrad
$\delta E/E$	0.2 %	$7x10^{-4}$	10^{-4}	nd.	10^{-4}

* 10 μM SPOT ASSUMED, ** COMPUTED FROM REPORTED EMITTANCE SPECIFICATION

VII. Current Capabilities

The design and performance of existing instruments similar to or relevant to the discussed microscope project are reviewed, before making recommendations for minimum performance characteristics required of the new instrument. These instrument parameters are summarized in Table 1.

1. The SLAC UED

The SLAC UED provides an example of a working MeV imaging instrument [37]. The UED operates at 180 Hz, providing ~100 fs bunches of 10^5 electrons at energies from 2-5 MeV. The heart of the instrument is a copy of the LCLS injector, which operates at high accelerating fields (130 MV/m). This produces a short pulse with good coherence properties, and has enabled ground breaking ultrafast studies on both gaseous and solid state samples. The SLAC UED has been used to study carbon-based materials for damage studies (Section VI.5), but the small transverse coherence and lack of a suitable sample environment has limited study of biological specimens so far.

2. The Madison Gun

Previously mentioned was the possibility for cryogenic superconducting accelerator technology to impact electron source design. One example of such an electron source is the University of Wisconsin-Madison superconducting RF gun [38,39]. The gun was designed for extremely high brightness applications such as soft-xray FELs. Employing liquid He-cooled superconducting magnets, the design specifications are for delivery of 200 pC, 70 fs bunches at 5 MHz, accelerated to 4 MeV with fields of ~30 MV/m. Such specifications indicate that superconducting RF sources can already achieve performances very close to the instrument envisioned in Section III.

3. The Dynamic Transmission Electron Microscope (DTEM) Project

The DTEM project currently at Pacific Northwest National Lab has aimed to address many of the same scientific challenges in biology as we lay out here, with a focus on μs-scale dynamics of biomolecules. The project has adopted a different approach, however, beginning with a commercial TEM (JEM-2100F/Cs) and modifying it to perform dynamic imaging [8]. The current "2^{nd} generation" instrument produces 10-1000 ns pulses of ~10^9 electrons using a UV photocathode, which are accelerated to 200 keV. These pulses are then transmitted down a standard microscope column, including a spherical aberration corrector, resulting in images with 1-10 nm resolution. Laser optics are available for pump-probe experiments.

The DTEM project has focused on producing single-pulse images, hence the high number of electrons per pulse. Indeed, this is necessary for single-shot pump-probe experiments. While single-shot pump-probe capabilities offer the widest range of possible scientific applications, this requirement is very strict. Note that, already, the 10 ns minimum pulse time of the DTEM is longer than the 1 ns required "native" tumbling time of a typical protein (Figure 1); such samples would need to be anchored or fixed in some fashion to be amenable to study in such an instrument. The goals of the instrument discussed in this paper do not rely on single-shot performance.

A primary lesson from the DTEM project is that optimizing beam coherence is essential to producing high resolution images. As discussed, high accelerating voltages aid in this pursuit. Careful, ground-up photocathode and accelerator design to produce and maintain low-emittance electron bunches is necessary. While economical, modified commercial equipment will limit the ability to implement optimal cathode and accelerator performance.

VIII. Required Machine Parameters

This section sketches the minimum machine parameters for an atomic-resolution capable high energy pulsed-source microscope. It is assumed that the final electron energy will be 1 MeV, the final desired resolution is 1 Å, and require 5 e- / $Å^2$ to generate interpretable images. Note this electron dose is close to what is used in modern cryoEM instruments, and is ~20x less than the Poisson-dictated Rose criterion for 1 Å-resolution image elements (100 photons/element ~ 10%

noise). We discuss the additional requirement such an image can be formed in 1 μs for dynamical imaging.

1. Objective Lens

Besides space-charge induced aberration, objective lens performance is the main factor determining microscope resolution. High energy (MeV) beams will require significantly stronger magnetic focusing optics than for keV beams. The relativistic wavelength of a 1 MeV electron is 0.87 pm, so a β = 5 mrad lens acceptance angle results in a maximum (diffraction-limited) resolving power of just over 1 Å. The aberration-limited resolution *d* of an incoherent imaging system can be estimated by [26]

$$d \approx \sqrt{C_s^2 \beta^6 + C_c^2 \beta^2 \delta^2}$$

For 1 Å resolution, β = 5 mrad, and beam energy spread $\delta = 10^{-4}$ we require C_s < 100 mm and C_c < 10 mm. For reference, a typical solenoid-style objective lens in a modern TEM typically exhibits an acceptance angle β ~ 10 mrad, C_s and C_c ~ 1.0 mm [20]. Aberration corrections, standard on top of the line instruments, further reduce C_s to just a few μm. Chromatic aberration is a major factor limiting resolution in modern TEMs.

Li and Musumeci have analyzed the construction of MeV TEM design, and proposed an objective lens design based on quadrupoles instead of the traditionally used solenoid [40]. Such as design theoretically has performance comparable to current objective lenses as judged by spherical and chromatic aberration coefficients (C_s = 95 mm and C_c = 48 mm). All state of the art Å-resolution capable TEMs employ spherical aberration correctors to reduce C_s, and the challenges of applying aberration correction technology to MeV beams has not been analyzed.

2. Electron Beam: Emittance, Current, and Pulse Structure

The critical parameters governing image resolution are the bandwidth and divergence of the electron beam at the sample. One must also get enough areal flux to the sample, so spot size and current are also important. A smaller spot size results in a larger divergence of the beam, which has fixed emittance, so these parameters are coupled.

To image a 10 μm spot at 1 Å / 10 μs resolution with a minimal 5 e / Å^2 dose, an average current of >100 μA is required. Previously, we considered an instrument delivering this current that could operate by providing bunches of 10^7 electrons accelerated to 1 MeV at 100 MHz. If the bunches are 100 ps long, then the space-charge density of these bunches is similar to that of the current SLAC UED, and 10x greater than in the DTEM (which operates at non-relativistic electron energies). Any configuration reaching 100 μA can be considered, however.

As discussed in the previous section, to achieve Å resolution with a lens comparable to those found in modern TEMs, we require an energy spread < 10^{-4} (*i.e.* 100 eV) and a beam divergence much less than the acceptance angle, << 5 mrad (*i.e.* 100 μrad, 2%). For a 10 μm spot, this results in a transverse emittance of ~ 1 nm-rad. For reference, the emittance of the

SLAC UED is estimated to be 3 nm-rad [X. Shen, manuscript in preparation], and the Madison gun delivers 200-1000 nm-rad bunches [39]. This is a strict requirement on the transverse emittance.

3. Stability

Engineering efforts to maximize the stability of the electron source and microscope optics must be a first concern. For example, simulations show in the design of pulsed microscopes, pulse-to-pulse energy jitter can cause significantly greater aberrations than comparably sized (but *stable*) energy bandwidth spread [41]. Any design review should include a detailed and quantitative analysis of the expected jitter in relevant components, and simulations to analyze how this will impact performance.

IX. Outlook and Recommendations

1. Technical Conclusions

By analyzing the fundamental physics involved and lessons learned from previous projects, it is possible to draw a few important conclusions. Regarding static imaging:

1. Current electron microscopes operate at less than their theoretical performance would indicate, and exactly why is unclear.
2. One possible way to push microscope performance would be to operate with a pulsed source at high voltage. This would
    a. facilitate ideal contrast transfer,
    b. enable jitter reduction via image alignment, and
    c. mitigate sample-induced chromatic aberration
3. A high-brightness, monochromatic source would enable highly coherent illumination that should improve image formation.

Regarding dynamic imaging:

1. Study of biological matter using a time-resolved TEM will be very challenging, as it is not realistic to acquire images in the tumbling time of a standard sample. A solution to this problem must be presented before pursuing such a project.
2. The performance of any time resolved TEM is limited by the beam current and emittance at the source. Therefore, source development should be the top area of investment in such a microscope.
3. Spatiotemporal resolution of any dynamic microscope is fundamentally (and severely) limited by space charge considerations. The only way to push this physical limit is to go to high accelerating energies. This is recommended.
4. A high-rep rate/quasi-DC electron source, while not optimal for any one single timescale, would enable flexible operation across a range of applications by enabling tradeoff between spatial and temporal resolution in a smooth fashion. The theoretical performance cost for this flexibility is small.

2. Assessment of Opportunity and Risk
Based on these conclusions, it is possible to evaluate the scientific goals discussed, to:

- Answer fundamental questions about the opportunities afforded by lensless (coherent) imaging *vs.* microscopy
- Improve the resolution limits of cryoEM to encompass the majority of the proteome at high resolution (~2 Å/30 kDa)
- Enable cryoET at nm resolutions for samples ~5x thicker than is currently possible (up to ~1 μm without aberration)
- Expand electron microscopy to address questions of biomolecular dynamics

Any such program should be designed to address these questions in a stepwise fashion to limit risk and learn lessons in a methodical fashion. The program would benefit greatly, however, from a long-term outlook – if the instruments designed so that it can be upgraded rather than re-built to achieve the next goal.

The author's conclusion is that pursuit of new electron source technology for EM is a worthwhile investment, but:

1. **A pulsed/MeV source should not be the only kind of source considered for static imaging of biological samples**. It may be the best option, but that is not clear at this time. Pulsed MeV sources address a number of issues, discussed, that *may* be the limiting factor in microscope performance. Until the precise limiting factor(s) is (are) identified, however, we cannot be certain a pulsed MeV microscope will improve performance. Of course, construction of the pulsed/MeV microscope is one way to determine the limiting factor(s).
2. At this time, **construction of a dynamical instrument for biological imaging does not seem to be a prudent investment** given issues with biomolecular tumbling. The science case in other fields may be strong enough to justify construction of such an instrument, but until sample environment concerns for biosamples are addressed, such an instrument should wait.

3. SLAC is an Ideal Location for Electron Source Development
SLAC is an ideal location to pursue such a program. Construction of an advanced electron microscope is an exercise in electron acceleration and beam manipulation. An electron microscope is a linear accelerator – one of SLAC's core competencies. With the construction of LCLS-II, SLAC is poised to be a world leader in both cryogenic and warm linac technology. Further, SLAC has deep expertise in the detector, vacuum, controls, and data acquisition expertise necessary to execute a complex microscopy program.

The ability of these core competencies to impact electron imaging science is reflected in the rapid success of the UED program at SLAC. The UED program provides a foundation for

understanding pulsed MeV electron instruments. The extension from diffraction to microscopy is natural.

Finally, SLAC has a budding cryoEM facility that is poised to be a world leader in electron microscopy research. The LCLS has already brought the bleeding edge of structural biology research to SLAC. Thus, SLAC is poised to become a hub of next-generation structural biology. Disruptive technologies, like MeV pulsed electron microscopes, would therefore have a willing base of early users at SLAC who could leverage the technology to produce high impact science on day 1 of operation.

Should SLAC become the site of a next-generation electron microscopy program, there are a few areas of expertise that should be developed at the lab to support the effort, in addition to the electron source design:

- **Direct Electron Detectors.** Research on electron detectors need not occur directly on-site, but should be conducted in a tight loop with microscope development. Ultimately a microscope will only be as good as the camera it projects images onto.
- **Phase Plate Technology.** Developing robust phase plate technology is necessary to utilize microscopes – current and future – to their full potential. Given SLAC's investment in commercial cryoEMs, it is already worthwhile considering investment in phase plate development research. Construction of an MeV microscope would strengthen this argument considerably.
- **Sample Environment.** Sample preparation and environment are crucial to producing good microscopy images. For biological samples, at cryogenic or ambient temperatures, sample preparation and environments present specific engineering challenges. Funding for such efforts should not be neglected, and the sample environment should be considered from the start of any microscopy program.
- **Computation**. Between the LCLS and new computer science division, SLAC houses the expertise to acquire and interpret the data from a new microscope. The quantity data obtained from the microscope will be significant, and it will be novel, however, so the ability to effectively turn images into information should be directly supported.

Finally, if an MeV microscopy program is pursued, SLAC *must* seek the expertise and advice of electron microscopists. While SLAC houses the technical competency to build an MeV microscope, there are no electron microscopists actively working at SLAC. The instrument described in this paper is radically different from previous designs, and this departure from the current thinking of the field should be retained, but it would be negligent to not assemble a small team of experts with experience building electron microscopes before pursuing this project.

The three grand challenges poised at the outset of this paper: routine imaging of non-crystalline proteins, molecularly resolved tomography, and dynamic imaging of biomacromolecules, seem certain to be realized sometime in the future. The impact of an instrument capable of meeting any one of these would be significant. Given the possibility that the next generation of electron microscopes could address all three, it is worthwhile to carefully consider what that next step will

be. Pulsed MeV instruments are one very promising route to improving the current generation of electron microscopes, and SLAC is uniquely positioned to pursue this line of research.

References

1. Khoshouei M, Radjainia M, Baumeister W, Danev R: **Cryo-EM structure of haemoglobin at 3.2 A determined with the Volta phase plate**. *bioRxiv* 2016, doi:10.1101/087841.

2. Piana S, Lindorff-Larsen K, Shaw DE: **Atomic-level description of ubiquitin folding.** *P Natl Acad Sci Usa* 2013, **110**:5915–5920.

3. Cosslett VE: **Current developments in high voltage electron microscopy.** *J Microsc* 1974, **100**:233–246.

4. Dupouy G: **Performance and applications of the Toulouse 3 million volt electron microscope.** *J Microsc* 1973, **97**:3–28.

5. Schwantes CR, McGibbon RT, Pande VS: **Perspective: Markov models for long-timescale biomolecular dynamics**. *J. Chem. Phys.* 2014, **141**:090901–8.

6. Ihee H, Lobastov VA, Gomez UM, Goodson BM, Srinivasan R, Ruan CY, Zewail AH: **Direct imaging of transient molecular structures with ultrafast diffraction.** *Science* 2001, **291**:458–462.

7. Li RK, Reid AH, Weathersby SP: **Development of MeV Ultrafast Electron Scattering Instruments at SLAC National Accelerator Laboratory**. *Microscopy and …* 2015, doi:10.1017/S1431927615006832.

8. Evans JE, Browning ND: **Enabling direct nanoscale observations of biological reactions with dynamic TEM**. *Microscopy (Tokyo)* 2013, **62**:147–156.

9. Henderson R: **The potential and limitations of neutrons, electrons and X-rays for atomic resolution microscopy of unstained biological molecules.** *Quart. Rev. Biophys.* 1995, **28**:171–193.

10. Hall E, Stemmer S, Zheng H, Zhu Y, Maracas G: **Future of Electron Scattering and Diffraction**. 2014, [no volume].

11. Aquila A, Barty A, Bostedt C, Boutet S, Carini G, dePonte D, Drell P, Doniach S, Downing KH, Earnest T, et al.: **The linac coherent light source single particle imaging road map**. *Struct. Dyn.* 2015, **2**:041701–14.

12. Egerton RF: **Outrun radiation damage with electrons?** *Advanced Structural and Chemical Imaging* 2015, doi:10.1186/s40679-014-0001-3.

13. Clark JN, Huang X, Harder R, Robinson IK: **High-resolution three-dimensional partially coherent diffraction imaging**. *Nature Communications* 1AD, **3**:993–6.

14. Marchesini S: **A unified evaluation of iterative projection algorithms for phase retrieval**. *Rev. Sci. Instrum.* 2007, **78**:011301–11.

15. Humphry MJ, Kraus B, Hurst AC, Maiden AM, Rodenburg JM: **Ptychographic electron microscopy using high-angle dark-field scattering for sub-nanometre resolution imaging**. *Nature Communications* 1AD, **3**:730–7.

16. Putkunz CT, D'Alfonso AJ, Morgan AJ, Weyland M, Dwyer C, Bourgeois L, Etheridge J, Roberts A, Scholten RE, Nugent KA, et al.: **Atom-Scale Ptychographic Electron Diffractive Imaging of Boron Nitride Cones**. *Phys. Rev. Lett.* 2012, **108**:073901–4.

17. De Caro L, Carlino E, Caputo G, Cozzoli PD, Giannini C: **Electron diffractive imaging of oxygen atoms in nanocrystals at sub-a ̊ngstro ̈m resolution**. *Nature Nanotechnology* 2010, **5**:360–365.

18. Glaeser RM, Hall RJ: **Reaching the Information Limit in Cryo-EM of Biological Macromolecules: Experimental Aspects**. *Biophysj* 2011, **100**:2331–2337.

19. Glaeser RM: **How good can cryo-EM become?** *Nat Meth* 2016, **13**:28–32.

20. Hetherington C: **Aberration correction for TEM**. *Materials Today* 2004, **7**:50–55.

21. Hawkes PW: **Aberration correction past and present**. *Philosophical Transactions of the Royal Society A: Mathematical, Physical and Engineering Sciences* 2009, **367**:3637–3664.

22. Cohen HA, Schmid MF, Chiu W: **Estimates of validity of projection approximation for three-dimensional reconstructions at high resolution.** *Ultramicroscopy* 1984, **14**:219–226.

23. Danev R, Buijsse B, Khoshouei M, Plitzko JM, Baumeister W: **Volta potential phase plate for in-focus phase contrast transmission electron microscopy**. *Proceedings of the National Academy of Sciences* 2014, **111**:15635–15640.

24. Danev R, Baumeister W: **Cryo-EM single particle analysis with the Volta phase plate**. *eLife* 2016, doi:10.7554/eLife.13046.001.

25. Chang W-H, Chiu MTK, Chen C-Y, Yen C-F, Lin Y-C, Weng Y-P, Chang J-C, Wu Y-M, Cheng H, Fu J, et al.: **Zernike Phase Plate Cryoelectron Microscopy Facilitates Single Particle Analysis of Unstained Asymmetric Protein Complexes**. *Structure/Folding and Design* 2010, **18**:17–27.

26. Scherzer O: **The Theoretical Resolution Limit of the Electron Microscope**. *J. Appl. Phys.* 1949, **20**:20–29.

27. Brilot AF, Chen JZ, Cheng A, Pan J, Harrison SC, Potter CS, Carragher B, Henderson R, Grigorieff N: **Beam-induced motion of vitrified specimen on holey carbon film**. *Journal of Structural Biology* 2012, **177**:630–637.

28. Shigematsu H, Sigworth FJ: **Noise models and cryo-EM drift correction with a direct-electron camera**. *Ultramicroscopy* 2013, **131**:61–69.

29. Rubinstein JL, Brubaker MA: **Alignment of cryo-EM movies of individual particles by optimization of image translations**. *arXiv* 2014, **q-bio.QM**.

30. Glauert AM: **The high voltage electron microscope in biology.** *J. Cell Biol.* 1974, **63**:717–748.

31. DIEBOLDER CA, KOSTER AJ, KONING RI: **Pushing the resolution limits in cryo electron tomography of biological structures.** *J Microsc* 2012, **248**:1–5.

32. Binshtein E, Ohi MD: **Cryo-Electron Microscopy and the Amazing Race to Atomic Resolution**. *Biochemistry* 2015, **54**:3133–3141.

33. Battaglia M, Contarato D, Denes P, Doering D, Duden T, Krieger B, Giubilato P, Gnani D, Radmilovic V: **Characterisation of a CMOS active pixel sensor for use in the TEAM microscope**. *Nuclear Instruments and Methods in Physics Research Section A: Accelerators, Spectrometers, Detectors and Associated Equipment* 2010, **622**:669–677.

34. Thomas G, Lacaze JC: **Transmission electron microscopy at 2.5 MeV**. *J Microsc* 1973, [no volume].

35. Chen JZ, Sachse C, Xu C, Mielke T, Spahn CMT, Grigorieff N: **A dose-rate effect in single-particle electron microscopy.** *Journal of Structural Biology* 2008, **161**:92–100.

36. Uhlemann S, Müller H, Hartel P, Zach J, Haider M: **Thermal magnetic field noise limits resolution in transmission electron microscopy.** *Phys. Rev. Lett.* 2013, **111**:046101.

37. Weathersby SP, Brown G, Centurion M, Chase TF, Coffee R, Corbett J, Eichner JP, Frisch JC, Fry AR, Gühr M, et al.: **Mega-electron-volt ultrafast electron diffraction at SLAC National Accelerator Laboratory**. *Rev. Sci. Instrum.* 2015, **86**:073702–8.

38. Legg R, Bisognano JJ, Bissen M: **Status of the Wisconsin SRF gun**. *Proceeding of this …* 2012, [no volume].

39. Bisognano J, Bissen M, Bosch R, Efremov M: **Wisconsin SRF Electron Gun Commissioning**. *…* 2013, [no volume].

40. Li RK, Musumeci P: **Single-Shot MeV Transmission Electron Microscopy with Picosecond Temporal Resolution**. *Phys. Rev. Applied* 2014, **2**:024003.

41. Xiang D, Fu F, Zhang J, Huang X, Wang L, Wang X, Wan W: **Accelerator-based single-shot ultrafast transmission electron microscope with picosecond temporal resolution and nanometer spatial resolution**. *Nuclear Inst. and Methods in Physics Research, A* 2014, **759**:74–82.