I. Executive Summary.

The world's first "hard" x-ray laser commenced operation at the SLAC National Accelerator Laboratory in Fall 2009.[1] "Hard" x-rays have a wavelength comparable to atomic dimensions (circa 0.1 nm) and they penetrate deeply through solid matter. Since the spatial resolution of a photon-based measurement is set by the wavelength, scattering of such x-rays can deliver atomically resolved, three-dimensional structural information on solid objects many tens of micrometers thick. The new laser was immediately employed for biological structure determination, with seminal diffraction measurements on Photosystem I (PSI) nanocrystals being carried out in Dec 2009.[2] The SLAC x-ray laser is a "Free-Electron Laser" (FEL) in which the lasing medium is a beam of electrons accelerated in a kilometer-long linear accelerator to nearly the speed of light. The electrons are made to oscillate by being passed through a magnetic field of periodically alternating polarity (an "undulator") and this induces emission of x-ray photons. Non-linear coupling of the photon field to the electron beam causes this photon emission to be coherent - the hallmark of any laser. By its nature a FEL operates in pulsed mode, indeed delivering extraordinarily short pulses, down to 10 fs ($10x10^{-15}$ s!). This is much shorter than the time scale for any physical motion (rotation, vibration) and so scattering of this pulse delivers a true "snapshot" of the sample, thereby greatly simplifying data analysis. On the other hand each pulse contains an astonishing 10^{12}-10^{13} photons and a correspondingly enormous energy flux. Consequently the x-ray beam, when focused to a micron-sized spot, completely vaporizes any solid material it encounters.[3] Nonetheless diffraction measurements are still possible by virtue of the extremely short pulse duration: Long before the vaporizing specimen actually flies apart, diffracted x-ray photons are already on their way to the detector, carrying with them a latent x-ray diffraction image of the essentially undisturbed specimen. This "diffraction before destruction" had been conjectured theoretically, but was only verified experimentally with the Dec 2009 measurements.

Absolutely critical to all FEL measurements, especially on biological species, is delivery of the sample specimen into the FEL beam. A biospecies of interest must be transported, fully solvated and at high concentration in its required buffer solution, into a micron-sized x-ray focal spot at a rate commensurate with the 120 Hz arrival rate of the x-ray pulses. This injection must continue uninterrupted for hours at a time. The Gas Dynamic Virtual Nozzle (GDVN)[4] sample injector previously developed by Bruce Doak employs a convergent coaxial gas flow rather than a solid nozzle to form a micron-sized linear liquid filament containing the sample species within an appropriate chemical solution. This "virtual nozzle" is largely immune to nozzle clogging and so delivers the requisite reliability. Virtually all FEL-based biological structure determination to date has made use of GDVN injectors. Nonetheless it was clear from the onset that improvements were not only possible but highly desirable. Principle among these was the need to reduce the amount of sample required. Current GDVN's deliver a <u>continuous</u> liquid free-stream to an x-ray beam that is <u>pulsed</u> at 120 Hz. Given a sample flow speed of typically 10 m/s, the liquid jet moves 8.3 cm between pulses. With an FEL x-ray beam focused to a spot size of 1 μm^2, the efficiency of sample usage is thus at best $1.2x10^{-5}$! The goal of this IIF research was to dramatically improve efficiency of sample use. Two separate approaches were eventually taken: A. Controllable

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[1] The Linac Coherent Light Source (LCLS) at the SLAC National Accelerator Laboratory, Menlo Park, CA.
[2] H.N. Chapman, et al., "Femtosecond x-ray nanocrystallography," Nature 470, 73-77 (2011).
[3] On one occasion, 10 μm diameter holes were unintentionally drilled through 3 mm of stainless steel.
[4] U. Weierstall, et al., "Injector for scattering from solvated species," Rev. Sci. Instrum. 83, 035108 (2012). See also United States Patent 8,272,576, R.B. Doak, et al., September 25, 2012.

intermittent use of a GDVN was demonstrated for the first time. B. The flow speed of the sample stream was dramatically decreased by use of a high viscosity medium. Both approaches proved quite successful.

A. Experimental measurements demonstrated the feasibility of intermittent injection into vacuum of a microscopic, linear, liquid free-stream from a Gas Dynamic Virtual Nozzle (GDVN). After a brief initial "turn-on" transient of ~10 microseconds, the intermittent stream appears to have all of the same advantageous attributes as that from a continuous-flow GDVN[4]. However the flow remains "on" for only a few tens of microseconds, after which it terminates abruptly and cleanly when operating in the proper flow regime. The duration ("on time") of the liquid stream never exceeds a few tens of microseconds and this can be varied by tailoring the geometry of the GDVN nozzle (bore diameter of inner capillary and distance of this capillary's exit from GDVN exit aperture), altering the dimensions of the GDVN meniscus and thereby the "on time" duration. The time between liquid stream emissions ("off time") varies much more dramatically and depends on both the pressure applied to the liquid in the inner GDVN capillary and on the pressure applied to the coaxially flowing GDVN gas in the outer capillary. We have demonstrated "off times" ranging from a few tens of *micro*seconds up to over 10 *milli*seconds. The latter are achieved at very low liquid flow rates. At 200 nl/min, for example, with no pressure applied to the liquid and 300 psi on the GDVN gas, a conventional GDVN[5] of 50 μm ID inner capillary emits an intermittent linear stream every 10.5 ms. The usable duration of these intermittent liquid free-stream spurts is about 35 μs and the usable overall length of the emitted spurt is about 350 μm. These attributes are ideally suited to intermittent delivery of sample-containing liquid streams at the repetition rate of the SLAC Linac Coherent Light Source (LCLS), namely at 120 Hz x-ray pulse rate (giving a pulse-to-pulse separation of 8.33 ms). Efficiency of sample use is increased by four orders of magnitude.

B . High-throughput x-ray diffraction data was collected in air at 1 atm as microscopic macromolecular crystals were passed at room temperature through an unattended third generation synchrotron x-ray beam. Specifically, diffraction images from serial x-ray exposures of 100 ms duration were recorded at ten per second (still images) or one per second (rotation images) from micron-sized lysozyme crystals embedded in a 35 μm diameter fluid free-stream of lipidic cubic phase (LCP), flowing at 500 μm/s orthogonally through a 10 x 30 μm x-ray spot of the Swiss Light Source (SLS) synchrotron. At constant sample flow under these conditions, each x-ray exposure probed a pristine volume of sample. For rotation-data acquisition (as is standard practice in synchrotron diffraction measurements), the LCP stream was rotated 0.2° about its axis during each exposure. The diffraction patterns showed diffraction to high angles and the integrated diffraction intensities compared well with conventionally acquired data. Background x-ray scattering from the carrier medium caused no problems. Critical to the success of these measurements were the extremely low speed of the LCP free-stream and its high viscosity. It was unknown, prior to this seminal demonstration, whether a combination of flow speed, duration of serial x-ray exposure, and exposure repetition rate could be found such that serial snapshot data recorded at a synchrotron would be of sufficiently high quality and quantity to allow extraction of a biomolecular structure. Accordingly, this study demonstrated seminal feasibility of both an apparatus and a methodology for room temperature biological structure determination at up to 10 Hz shutterless serial synchrotron x-ray diffraction from randomly oriented biological microcrystals carried in a flowing microscopic stream.

[5] "Conventional GDVN" here denotes one in which the cusped GDVN meniscus from the inner capillary is fully contained within the outer envelope through which the GDVN gas passes.