SINGLE-PARTICLE IMAGING

D. Sayre
Dept. of Physics & Astronomy, Stony Brook University, Stony Brook NY 11794 USA

The subject of this talk is the imaging of general small objects by essentially the same methods as are used today for the imaging of unit cells of crystals; it could thus be called "crystallography without need for a crystal". Here is the point that had been reached in the subject a few years ago; it is taken from an article in Nature in 1999 published by our group at Stony Brook. The "small object" looked like this [1] under an SEM. Its diffraction pattern when placed in the x-ray beam looked like this [2], and the image produced (i.e. the Fourier transform of the pattern after phasing) looked like this [3]. Notice [2] that the diffraction pattern was not concentrated into discrete Bragg spots, as it would have been if the object had been repeated many times on a lattice. It was much weaker than that, and required more intense exposure to be recorded, but being continuous (and I'll show another picture in a minute which will let you see the continuity much better), and not having lost the information *between* the Bragg points, it carried more information -- enough more in fact that it only took a rough knowledge of the *envelope* of the object to allow the phases to be quite easily determined. I will go into the method of phasing in more detail later.

Today we have advanced considerably, in fact to the imaging of a whole biological cell, a *yeast* cell, recently living, but then plunged into liquid ethane, and then brought up through temperature stages to sublimate out its water content and become at room temperature a freeze-dried cell. Many of its fellow cells, when put back in water, resume living. *Its* diffraction pattern, taken with the specimen sitting stationary in a 16.5A-wavelength x-ray beam at the Lawrence Berkeley ALS by David Shapiro (at that time a StonyBrook graduate student) -- and this is David Shapiro -- looks like this [4]. You can see the continuity of the pattern. And the transform of the pattern, with the phasing mainly worked out by Prof. Veit Elser at Cornell and his graduate student Pierre Thibault, looks like this [5]. Here [4'] is a closer-up view of the pattern, and you can see unmistakeably that the intensity is gathered into speckles but is not discrete. Here [5'] is a closer-up view of the *image*. The colorization here is genuine, in the sense that intensity and hue code for the magnitude and phase of the cell's scattering density which, at these softer photon energies, is complex, and which in future *3*D maps will help identify local composition, chemical state, and specific feature markers which may have been introduced. Later David will be showing this and related images more fully, but for now let me point out the scale bar and the areas of enlargement, which tell us that the cell is about 3 microns in diameter, and that there is repeatable detail in it down to the 30nm size-level. (The two side-by-side pictures show the cell in two orientations 1 degree apart, and note that the same detail still appears.) Thus, since 30nm is about the size of an individual ribosome, this slide suggests that the day is not far off when a *three*-dimensional image like this will allow us to see the actual position of every object of ribosome or larger size in a cell, and to do so in a cell that was living up to the moment of its freezing. David's data *actually* go out to about

***9*nm resolution, and in the full *3*-dimensional imaging of the *3*-dimensional data the imaging will *not* be somewhat blurred, as it is *here*, by its being a projection of the full thickness of the cell. Genuine *3-dimensional* imaging is thus the next major goal of our group, and our work thus far indicates that there should be no major impediment to success. I will also be discussing *that* later in the talk. What might be the impact of such imaging in biology? It is reasonable to think that, just as in *crystallography*, where knowledge of *atomic* positions and movements has given us a detailed understanding of the actions of large bio*molecular* entities, knowledge of bio*molecular* positions and movements could lead to a similar understanding of the actions of entire *cells*. Stated differently, crystallography may be able to contribute to *cell* biology what it currently contributes to *molecular* biology.**

This work on the cell is the work which *our* group is doing, but since the Nature article a lot more is now going on. The methodology today is getting to be known as x-ray single-particle imaging, or x-ray diffraction microscopy, and people now are working on it in a dozen or more institutions around the world, including StonyBrook and Cornell, Illinois, Stanford, Berkeley, Arizona State (the Arizona group is led by Prof. John Spence, who is chairing this session), Livermore, and UCLA; Uppsala in Sweden, Hamburg and Berlin in Germany, and SPring8 in Japan, and maybe others that I don't kmow of. In some places the emphasis is on materials science specimens; in other places, work has turned to the possibility of single-particle imaging of biological macro*molecules* and macromolecular assemblies. Thus, single-particle activity now is starting to exist in materials science areas, and in biomolecular and biocellular areas. Let me remind you too that this afternoon, a fuller microsymposium on the subject than just this talk (MS22) will be taking place, organized by Janos Hajdu of Uppsala and Henry Chapman of Lawrence Livermore.

****

With that introduction, in this next section let me talk in more detail about the *carrying-out* of single-particle imaging. Everyone here is familiar with how *crystallography* is done, so the simplest thing to do will be to bring out the major *differences* which exist; and I will *do* that, under the three main headings of getting the specimen, getting the intensities, and getting the phases.

First, getting the specimen and mounting it for exposure. Here is where the first big difference comes, in that getting a good crystal is not required. Take a cell, or a molecule, or *any* tiny fragment of matter, and it will be a fine specimen. This can bring a very large saving of time, and at the same time a large opening-up of new scientific application areas. It can be a problem also. For example, in our yeast cell work, the weakness of the scattering from the tiny non-crystalline specimen makes it desirable that the amount of specimen *mounting* material in the beam be kept to a minimum -- a carbon nanofiber mount would be good, for example, or a microcapillary with nanothin carbon walls. Currently in our work, however, we have still not gotten away from the standard planar geometry of a nanothin membrane supported on an EM grid, with the unwanted effect that when the grid is

edge-on to the beam it blocks transmission and prevents access to some of the 3-dimensional data. (Fortunately, the phasing technique can go some distance in supplying that missing data.) In the bio*molecular* case, where there can be unlimited exact *copies* of the specimen, and where the planned technique is built around using those copies to collect a very large number of flash diffraction patterns with extremely brief extremely intense x-ray pulses from an x-ray laser, molecular spraying techniques can give an entirely *mountless* method of getting a single molecule briefly into position for the femtosecond beam flash. That is very good, but of course raises the familiar problem of knowing, for each pattern obtained, what the molecular orientation was for that pattern; however, that problem is solvable provided the photons/pulse are sufficient to keep the Poisson noise level reasonable in the individual patterns. In summary, in obtaining specimens, single-particle work gives an extremely large increase in ease and range. But with a few new specimen-handling problems as well.

Next, exposure and radiation damage. This is where *crystallography* is particularly strong, due to the large reduction in x-ray exposure given by the signal amplification at the Bragg spots, and due also to the sharing of damage over the many copies of the object being imaged. The *amplification* is lost in single-particle work. Still, in biomolecular work, where the many exact *copies* do exist, the x-ray flash method, when it is perfected, should image at or near atomic resolution, and it is important to realize that it can do that *independently* of whether the many copies can be persuaded to form a *crystal* or not. In the biological *cell* case today, and in many materials science cases, where exact copies are *not* available, that ability to *do* the imaging still holds, and at least in materials work, where specimens can often be quite radiation resistant, the imaging may again reach atomic or near-atomic resolution. Coming however finally to the bio*cellular* case, unless a method of generating a population of cells identical all the way to the atomic level should someday become available, imaging resolution must depend upon how long a single cell, once mounted, can continue to diffract consistently in the x-ray beam. Fortunately, resistance of a biocell to damage can be increased through a number of techniques, of which the least invasive is instant fixation by fast freezing, followed if desired by the addition of chemical fixatives as well as of specific site markers, etc. And here, in our work on the yeast cell, we find that fast freezing, either by itself or followed by freeze-drying, gives enough radiation resistance to allow the collection of hundreds of good 10 nm resolution diffraction patterns from one cell; this means that, for the *yeast* cell at least, there should be no fundamental barrier to that level of 3-dimensional imaging. Stated differently, until a few months ago it was possible that we would find that a cell in the beam could give a pattern, but could not survive in the beam long enough to give the *many* patterns necessary for its imaging in 3 dimensions, but our recent work -- in reality mostly David's work -- has told us that, for yeast at least, that is not the case.

Third, phasing and image reconstruction; here too is an area where single particles do very well. In the *crystal* case the transform of the unit cell is effectively only observable at the Bragg points, whereas in the *single-particle* case the transform, though weak, is continuous and can be observed at any desired fineness. And *with*

the finer sampling, provided that a fairly good *envelope* of the particle is known, the phase problem almost disappears. It takes quite a lot of computation to *make* it disappear, but it does effectively disappear. This story developed over a number of years. It began with a paper, based on Shannon's theorem, that I wrote in 1952 on what would happen if we had finer-than-crystallographic sampling; that was followed by the work of Gerchberg and Saxton in the 1970s based on fine-sampling of non-crystalline EM data, followed by the work in the optics literature of Bates and Fienup in the 1980s; then proposed in 1990 for the non-crystalline object in the *x-ray* case by Gerard Bricogne and myself, followed later in the '90s by the actual demonstrations by Henry Chapman and John Miao, and most recently now by Elser and Thibault at Cornell, also Stefano Marchesini et al. at Lawrence Livermore Laboratory. There are several ways of presenting the subject, but to keep things simple I will talk about it in regular crystallographic language. To arrive at the basic idea, imagine that we have sampled the magnitudes at more than Bragg fineness, and imagine also that we somehow have the correct phases. Fourier summing will then deliver the correct image repeated on a lattice larger than that which is necessary to keep the repeated images separate from each other, i.e. each image will be surrounded by a sea of zeroes. Given *incorrect* phases, however, some density will *escape* into what should be the sea of zeroes. Starting with *random* phases, then, go into image space, push all non-zero pixels outside the specimen envelope toward zero, come back into diffraction space, adopt the new *phases*, and repeat, until finally everything outside the envelope is zero; what is *inside* the envelope will be the correct structure. Here is an early demonstration of the process, which we published in Acta in 1998 [6]. Here is a finely-sampled dataset. Here is the initial transform of that dataset, with random starting phases. You can't see any structure. Here is the transform after 50 cycles of pushing down on the values outside the envelope. There is still a sprinkling of values which have not been fully pushed down. Here is the transform 50 cycles later -- the outside values now all look like zero -- but things are still evolving. Here is the transform 100 cycles later -- motion has now ceased, and the structure inside the envelope has reached its final and more meaningful form. And *here* is the structure which was used in the first place to generate the dataset. You can't distinguish it from the final transform. (The last two figures show what happened when noise was added to corrupt somewhat the initial dataset. It now took 425 cycles to reach a final structure, and it is now slightly visibly different from the transform of the changed magnitudes with the correct phases. The process is therefore fairly robust in the presence of experimental error.) Detailed issues of how large the zero sea should be, of the best ways to push toward zero, of enforcing possible constraints on the pixels *inside* the envelope, and of refining the envelope itself, have been worked on by Fienup and others, and especially recently in the "difference map" technique of Elser. Today the overall result is clear: given a reasonable initial knowledge of the envelope, reasonable sampling fineness, few sampling absences, reasonably small error in measuring the magnitudes, and plenty of computing power, the method is proving to be reliable, fast, and easy. So the phase problem, in single-particle work, is almost a pleasure to think about.

******

(Omitted, to save time, in the spoken version of the talk.)

That is a quick take-through on the underlying techniques. Now I would like to turn back to give a brief history of the StonyBrook/Cornell/ALS Berkeley project. This had its origins in the 1970s, and arose from the uncomfortable feeling that I had that crystallography must in time run out of crystals; I asked myself if there might be some way of letting x-rays image without crystals. In 1973-4 Janos Kirz at StonyBrook and I began by working on the idea of x-ray microscopes using ultrasmall Fresnel zoneplates; by 1979 Brookhaven had begun building its synchrotron and Janos was starting on the first of a sequence of microscopy beamlines and scanning transmission x-ray microscopes there; also in 1979 I wrote a short paper presenting the thought that the key ideas of crystallography itself might be made to work *without* crystals. Through the '80s and '90s Janos sheltered and encouraged the crystallography-without-crystals project, giving me photons and help of every kind, including almost always a graduate student or a postdoc. Those years saw the first two crucial questions -- could a synchrotron get a detectable diffraction pattern out of a single biological cell? and could that pattern be phased? -- answered in the affirmative, the first in 1987 by graduate student WenBing Yun, and the second starting in 1989 when Gerard Bricogne and I one day fished back in our memories to the paper I had written in 1952 saying that if crystals would only let us measure between the Bragg points, phasing would become much easier, and we realized that the *non*-crystal, which was what we wanted to work with anyway, was also the very thing that would indeed *get* us between the Bragg points. I put that in a talk given at Erice in 1990, and in 1995 postdoc Henry Chapman, who was familiar with Fienup's hybrid input-output work, and in 1998 graduate student John Miao, then did actually *demonstrate* the hoped-for phasing. Then finally, in 1999, John Miao put the data-taking and the phasing together, and we did the experiment that appeared in Nature and that I showed at the start of this talk. I turned 75 at about that time, and Janos Kirz and younger faculty member Chris Jacobsen at Stony Brook took over the management of the project, and were successful in obtaining a research grant from the NIH to try the single-particle approach on a yeast cell. The crucial question now was whether the yeast cell could survive in the beam long enough to allow the measuring of a full 3-dimensional diffraction pattern. For this a new low-temperature apparatus was built; the work was moved out to Berkeley for more photon intensity; and last year David Shapiro, helped by graduate students Enju Lima and Huijie Miao, gave an affirmative answer to the survival-length question, and also embarked on the transition to 3-dimensional imaging with a small 9-pattern rotation-set of patterns taken at 1 degree rotation intervals. In the meantime, in 2002, I had become aware of the great strengthening that Veit Elser at Cornell was contributing to phasing technique, and in 2003-4 we started to work in earnest with him and his student Pierre Thibault, who have now taken over most of the phasing work

****

Let me turn now to where three-dimensional imaging stands today. In our cell work, a start has been made by David Shapiro and Pierre Thibault, with David obtaining not just the one stationary pattern that we showed at the outset, but a set

of 9 such patterns taken one degree apart in the cell orientation, and Pierre then going on to phase those patterns and obtain the 9 successive cellular views. He has put those into a little movie, which I will now ask David to show.

[David show movie and say a little more on colors, 30nm detail, internal consistency, foreground moving l to r and background r to l, fact that information for 3D is there.]

Note that in this work only one small group of 9 planes in diffraction space have been obtained and phased. Thus the step to a fully three-dimensional phased dataset has yet to be taken by us. At Lawrence Livermore it has now however been *beautifully* taken on two 3D *man*-made specimens, somewhat as in our *2D* specimen of 1999, by Henry Chapman and his colleagues, and I will ask David, who is *at* Lawrence Livermore now, to show a little movie of the earlier of those; the newer one will be shown this afternoon. [David show.]

****

So we see that progress on the two things that a person in 1999 would have wanted to see -- an advance to real *bio*specimens, and an advance to*3*-dimensional imaging -- *is* taking place. In an interesting development *we* in fact expect to image the yeast cell in *two* different ways. Let me explain.

A beamline at Berkeley is now being planned which will allow the production of photons at wavelengths of 8A and less, as well as in the present softer range which covers the so-called "water window" extending from about 23 to 44A. The 8A photons have sufficient penetrating power that the Born approximation will be met by our 3-micron yeast cells, and regular 3D phasing and imaging will be able to take place through the fine-sampling technique. This approach has the desirable property that as crystallogaphers and cell biologists begin to want to do single-particle imaging, and want to graduate from small yeast cells to larger (e.g. human) cells, it will only be necessary to shift to more penetrating (such as 5A- or 2.5A-wavelength) x-rays. (Such a start in shorter wavelength diffraction, using E. coli as the specimen, has recently been made at SPring8 in Japan by John Miao and his associates.) With the 23A photons the penetrating power is less, and the Born condition does not accurately hold, but Elser has pointed out that another condition, the Rytov approximation, which treats diffraction basically as a transmission rather than a scattering phenomenon, *does* still hold, and this leads to a different method of image formation, in which the phasing and imaging is carried out *2*-dimensionally, at each separate orientational setting of the specimen -- just as in the 9-setting movie which David showed -- and *then* brought together into 3-dimensional space to obtain the desired 3-dimensional image; that process is, in short, a tomographic, rather than a direct, 3D imaging method. The advantage of the softer-photon technique, biologically speaking, is that the vitreous ice in a flash-frozen cell can be left in *place* in the cell, providing the highest degree of protection of the original structure from radiation damage, while at the same time the ice, being highly transparent to the water-window photons, effectively *disappears* in the imaging, allowing the organic material to be displayed, in its full

complex-valuedness, with full clarity. Thus we think that water-window imaging may become a sort of specialty method for the smaller cell types and for individual cellular components, providing the highest quality of imaging, and as such *very* *worth* developing along with the more usual direct 3D type of imaging. We hope, in another year or two, to have 3D images of the yeast cell by both of these imaging methods. For me, and I hope for every crystallographer, that will be an exciting day.

****

That brings us close to the end of this talk. X-ray crystallogaphy is now almost a century old, and growing more vigorous every year, and wherever multiple perfect copies of an object can be made, and can be assembled into a crystal, it has built vital new science. This talk I hope has revealed that those requirements of multiple copies and crystallizeability are not really *necessary* in the methodology, and are, or soon will be, able to be treated as options rather than requirements in crystallography. And with that *understanding*, crystallography may be able now to look forward to starting on its second century with even *added* vitality, through its increased adaptation to the study of many new types of real materials and of difficult-to-crystallize biomolecules, and perhaps most of all for its ability to carry out imaging of the intact, almost-natural-state, biological cell.

Thank you for your attention. That brings us to the end of the talk.