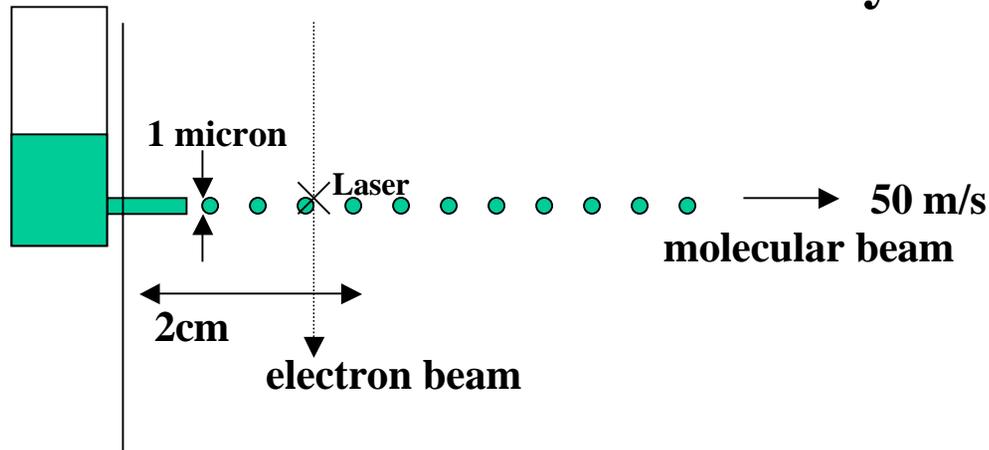


Serial Crystallography.



We* have built an apparatus to obtain electron diffraction patterns from a beam of vitreous ice balls, each containing a protein. Cryoprotectant. Molecules will be aligned by a polarized laser beam. All beams run *continuously*.

AIM: To solve protein structures which cannot be crystallized.

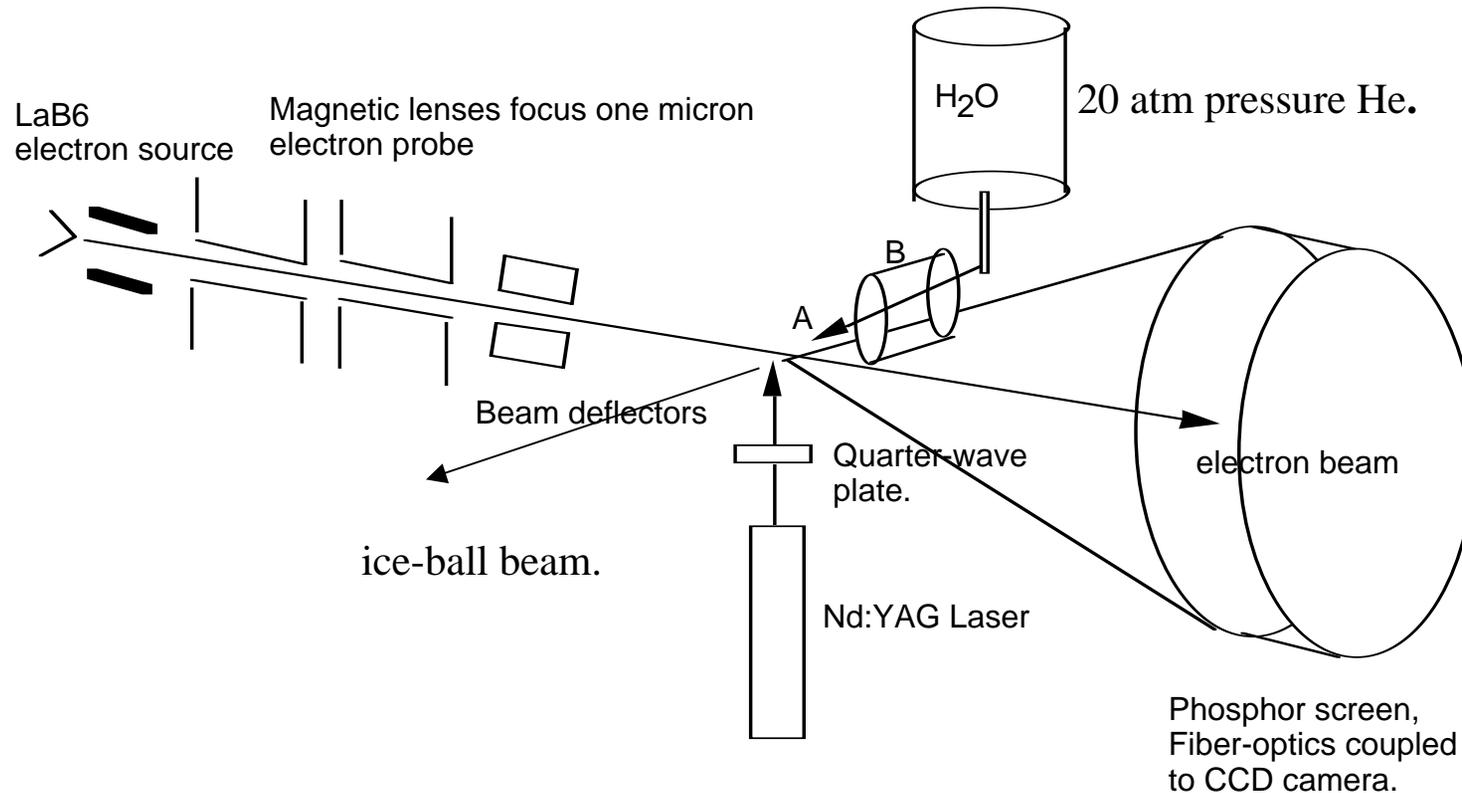
Spence and Doak, Phys Rev Letts. 92, 198102 (2004). Spence et al Acta A. Submitted.04

*Spence, Doak, Weierstall, Schmidt, Fromme, Hembree. ASU NSF SGER \$.

Structural Biology Background:

- *There are 33,000 human genes, coding for 1,000,000 proteins. (300,000 generations since LCA).
- *66,000 unique proteins have been solved by XRD. Consist mainly of C,O,H,N, size is 4 - 40nm
- *70% of drug mols interact with a membrane protein, only 83* of 300,000 mem prot have been solved, because they are very difficult to crystallize. (hydrophobic exterior)
- *Co-crystallization gives point of attachment of drug molecule.
- *Secondary structure of protein: 50% of a globular protein is alpha-helices and beta sheets.
80% of alpha helices can be predicted from sequence alone.
60% of beta-sheets can be predicted from sequence.
- *NMR (up to 30,000 Daltons ?) takes up to a year.
- *Few protein xtals diffract to atomic resolution. Fit templates from known amino acid structures ?
- *Amino acid (20) sequence is usually known, atomic structure of amino acids is known.
- *Amino acids can be distinguished at **0.3nm** resolution. Secondary structure at **0.7nm** (alpha helix).

**Nozzle expansion of water into vacuum can produce single-file ice balls by evaporative cooling (Faubel).
 Nozzle expansion of cold helium gas into vacuum produces superfluid helium at 0.4K (Toennies).
 Size of droplets is somewhat less than aperture diameter. Velocity > 30m/s. Twice diam between droplets**



Electron gun and lens forms ten micron probe at 10 - 40 kV. " B" is cryo-shroud to pump away water vapour. "A" is a submicron nozzle which produces a stream of water droplets which freeze to form vitreous ice balls.

*There are many oriented mols in the electron beam at one time. Coherence width of 10 micron beam is about size of mol, so no "speckle" interference between different mols.

***After 80 sec exposure of many mols in a single orientation, the electron diffraction pattern is read out.
 Then the laser polarization is rotated to a new orientation.**



Kimball Physics 50 kV LaB6 gun



**Main chamber:
2000 l/s diff pump
Water jet out of page
Laser down from top
Electron beam across from left**



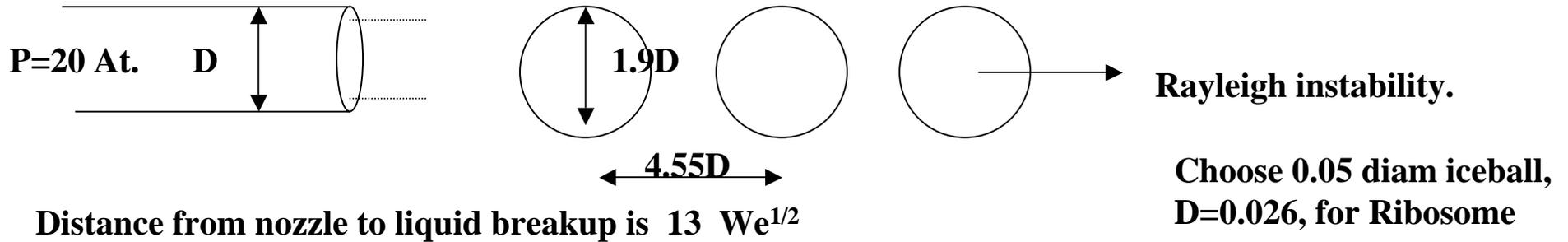
CCD camera for diff pattern



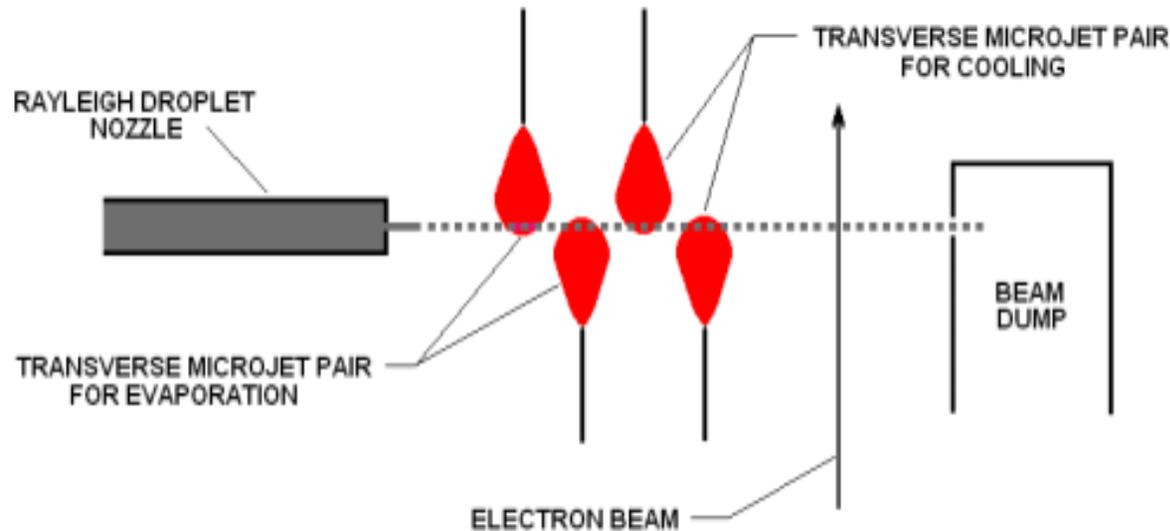
Water chamber at 20 at helium.

Rayleigh droplet jet. - surface energy minimization.

Under correct conditions, get single-file, equally spaced, identical droplets. Try $D = 0.03$ micron ?



Rayleigh, Proc Roy Soc A29,71 (1879); Frohn and Roth text, Faubel Z. Phys D10, p. 269 (1988)



**Doak iceball source. First jets of cold helium (or nitrogen) gas sublime ice to shrink iceballs
Second jets reduce temp to improve alignment for the smallest proteins.**

We is the Weber number, $We = \rho U^2 D / \sigma$ for a jet of density ρ and surface tension σ moving at speed U .

Condition for droplet formation.

From theory of laminar flow and turbulence, for water, we get

1. Droplets if $Re < 250$
2. Smooth columnar laminar stream if $250 < Re < 1200$
3. Diverging Spray if $Re > 1200$

$Re = \text{Reynolds number} = \frac{v r a}{h}$ for nozzle diam a , viscosity h .

Hence need nozzle diam $a < \frac{250 h}{v r} = \frac{250 * 1040E-6}{(50 * 1E3)}$

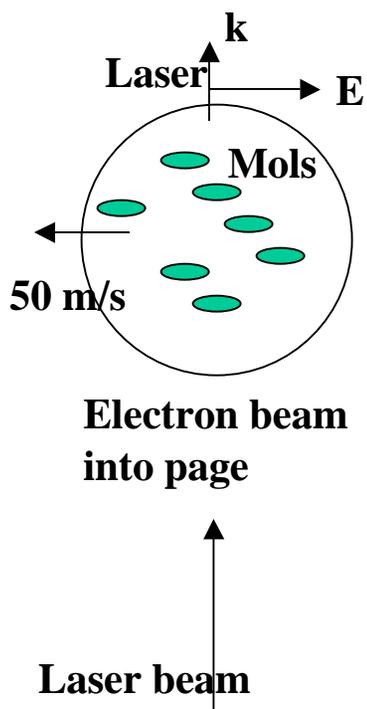
= 6 micron or less , for water droplet formation at $v = 50$ m/sec.

Cooling rate is about 10^6 K per second.

Velocity given by Bernouli: $v = (2 P/ r)^{1/2} = 45$ m/sec for 10 atm

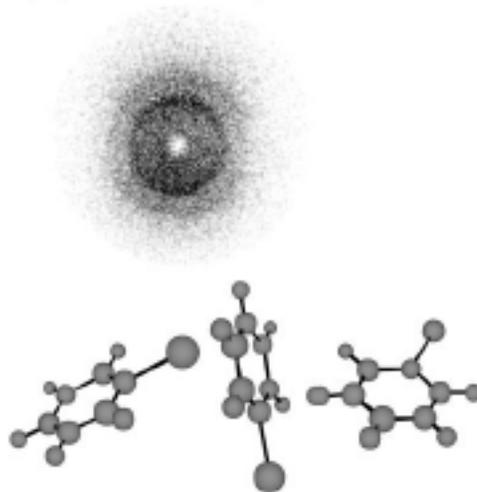
Rayleigh stability condition: Unstable for sound wavelengths $\lambda > \rho a$
(for $v = 50$ m/sec, $a = 5$ microns, critical freq is 2.9 MHz.)

Faubel Z. Phys D 10, p. 269 (1988).

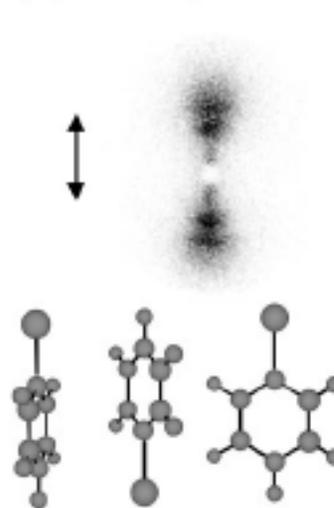


Laser alignment of molecular beams.

(a) No YAG pulse



(b) YAG pulse on



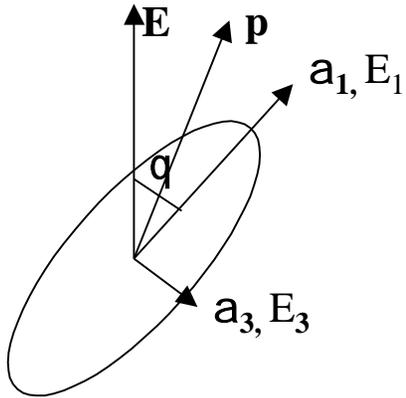
Molecules of Iodobenzene in a gas jet aligned by linearly polarized light from a YAG laser. After alignment, the mols are ionized and exploded by a more intense circularly polarized laser pulse, and the ions produce the experimental pattern shown. 3-D alignment using elliptically polarized light has since been demonstrated.

Larsen, J. Chem Phys 111, 7774 (1999).

Rotational temp 20 K

Mechanism of molecular alignment.

Induced dipole moment \mathbf{P} is not parallel to \mathbf{E} for tensor polarizability \mathbf{a} .



$$P_i(t) = a_{ij}(q) E_j(t)$$

$$\text{Torque is } \mathbf{T} = \mathbf{p} \times \mathbf{E}$$

$$\text{Energy change is } dU = -\mathbf{p} \cdot d\mathbf{E} = -p_3 dE_3 - p_1 dE_1$$

Now $p_3 = a_3 E_3$ and $p_1 = a_1 E_1$ for ellipsoid, where E_1 is cmpt along \mathbf{a}_1 .

$$\text{So } dU = -a_3 E_3 dE_3 - a_1 E_1 dE_1$$

Integrating,

$$U = -0.5 (a_3 E_3^2 + a_1 E_1^2)$$

With q between \mathbf{E} and molecular axis \mathbf{a}_1 ,

$$U = -0.5 (a_3 E^2 \cos^2 q + a_1 E^2 \sin^2 q)$$

$$U = -0.5 a_1 E^2 - 0.5 (a_3 - a_1) E^2 \cos^2(q)$$

Molecular alignment in laser field.

$\mathbf{p} = \mathbf{a} \mathbf{E}$ defines \mathbf{a} , for induced dipole moment \mathbf{p}

$$H = H_0 - \mathbf{m} \cdot \mathbf{E} - \frac{1}{2} \mathbf{E} \cdot \mathbf{a} \cdot \mathbf{E} - \dots$$

Second term (linear Stark) is zero for AC field. Third is sensitive only to RMS, hence can use laser to get large time-average field. Then, with $D\mathbf{a} = a_{zz} - a_{yy}$ and q angle between $\mathbf{E} = \mathbf{E}_z$ and mol axis,

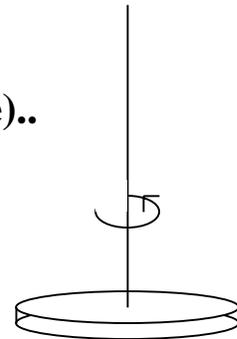
$$H = -\frac{1}{4} \hat{A}_{r,r'} E_r a_{r,r'} E_{r'} = -\frac{1}{4} E^2 D a \cos^2 q - c + \frac{1}{4} E^2 D a q^2$$

for symmetric top mol with plane-polarized light, giving one-dimensional alignment. Here $\mathbf{r} = (x,y,z)$ are space-fixed coords, \mathbf{a} a tensor. E^2 involves DC and freq doubling.

This is a "**torsion pendulum**" for small oscillations. Minimum energy for $q = 0$.

Spring constant $k = 0.5 E^2 D a$

Elliptical polarization fixes orientation about all axes ? (elliptical = plane + circle)..



DC Polarizability a of proteins.

Use DC values away from resonance.

Treat protein as dielectric ellipsoid. For spheroid, $a=b$. Prolate (cigar), oblate (pancake)

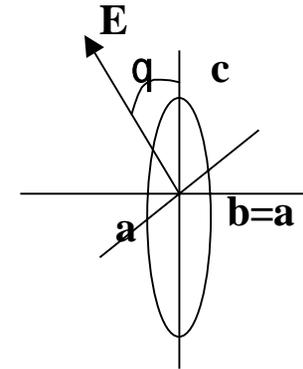
Assume prolate with eccentricity $e = 0$ (sphere), $= 1$ needle.

Choose $e = 0.8$, fixing $L_z = 0.2$ and $L_{xx} = (1 - 0.2)/2 = 0.4$. Then

$$a_z = V \frac{K - 1}{3 + 3L_z(K - 1)}$$

$$a_{yy} = a_{xx} = V \frac{K - 1}{3 + 3L_{xx}(K - 1)}$$

$$Da = a_z - a_{xx}$$



where K is dielectric constant for protein, V is volume $= (4\pi/3) abc$.

For Lysozyme, $K = 15$ if hydrated (experimental, Simonson '02. For water $K = 80$).

Then, in cgs, $Da = 0.63 \text{ Volume}$ (or $Da = 0.3 \text{ Vol}$ if $K = 4$, not hydrated).

This is **shape anisotropy**. 0.63 depends on both dielectric const and eccentricity

Phage virus is $30\text{nm} \times 300 \text{nm}$.

Note: Other sources of anisotropy to increase Da and increase alignment:

*vibrational, near resonance (a is a tensor)

*Attach small molecule to increase shape anisotropy.

Laser alignment of large molecules at low temperature.

For a molecule of length L, the difference in polarizability along two major axes is, in cgs units, $Da = g L^3$, where $g \sim 0.1$ is a constant (spheroid, classical, non-resonant). Proportional to **volume**.

$H = -\mathbf{m} \cdot \mathbf{E} - 0.5 \mathbf{E} \cdot \mathbf{a} \cdot \mathbf{E}$ (cf anisotropic polarizability effect on Stark spectra, nonlinear optics)

The interaction energy of a polarized laser beam of power P Watts/cm² with molecule is

$$H = H_{rot} - \frac{1}{4} \hat{\mathbf{A}}_{R,R'} E_R a_{R,R'} E_{R'} \quad \text{For linear mol in linear polarized field (} a_x = a_y \text{ etc),}$$

$$\mathbf{H} = -0.5 |\mathbf{E}_{rms}^2| Da \cos^2 q = -2p (P/c) g L^3 a q^2 \quad \text{for small angles.}$$

Setting $H = 0.5 kT$, with Da in nm³, P in watt/cm², this gives

$$\langle J^2 \rangle = \frac{T}{3 \times 10^{-8} P Da}$$

RMS angular displacement is $q_{rms} = \text{sqrt}(\langle q^2 \rangle)$

$$= 6 \text{ degrees} \quad \text{if } T = 4 \text{ K, } P = 10^8 \text{ Watt/cm}^2, \quad L = 10 \text{ nm}$$

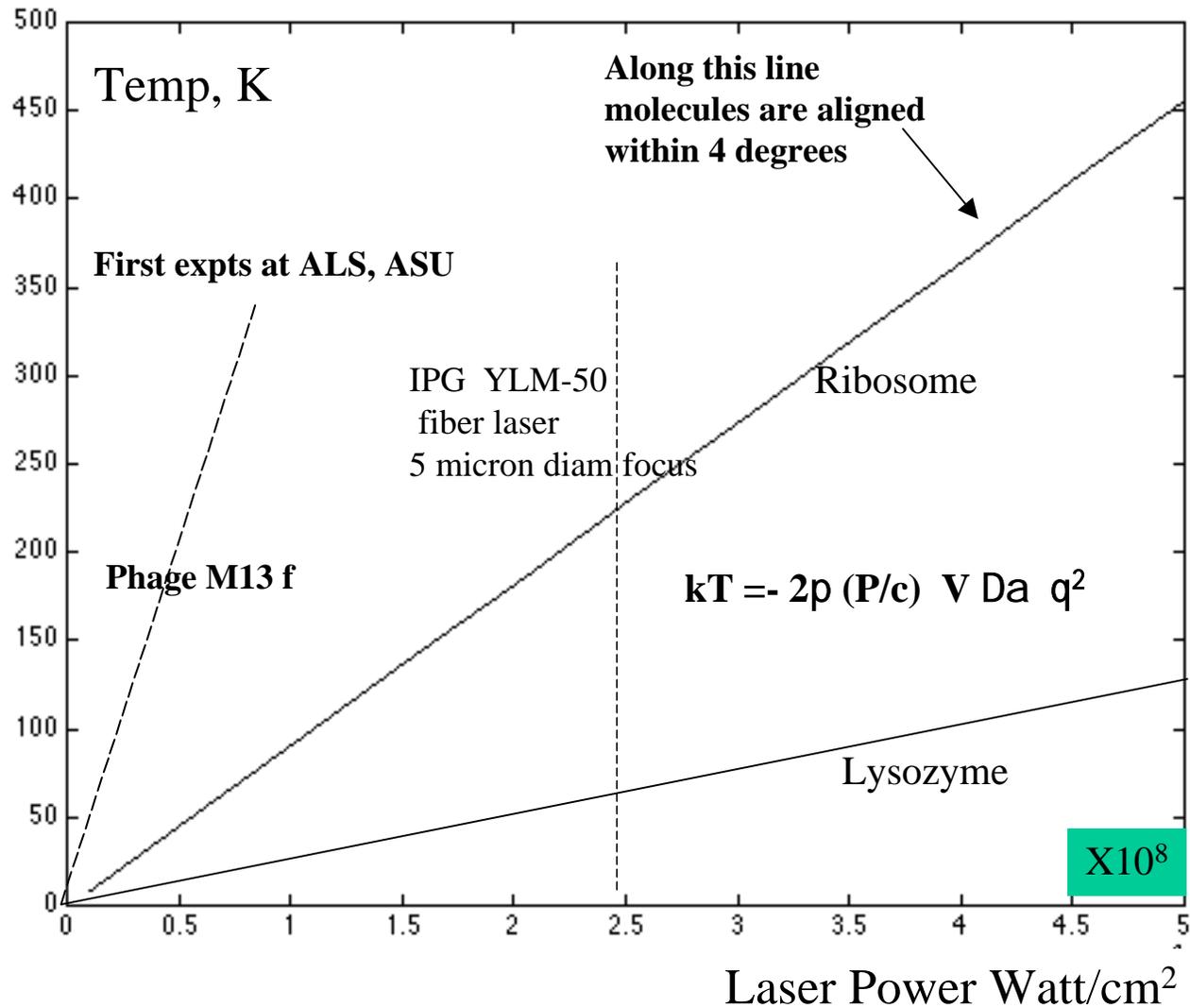
(= 1.8 deg for the larger Ribosome at 4K if $g=0.63$ and $P=10^8$)

The resolution limit is then $d = L q_{rms} = 1 \text{ nm}$.

Conclude: Alignment error is **propn to temp, inversely to molecular volume, laser power, anisotropy.**

Need large *difference* in polarizability along two axes. Phage virus is 30nm X 300 nm.

Laser power and temperature requirements for good diffraction patterns.



Temp (K) vs laser power in watt cm⁻² for Ribosome, Lysozyme, cylindrical Virus Phage
 Radius of ribosome 17nm. Radius of Lysozyme 11nm. Misalignment range fixed at 4 degrees
 g (anisotropy) = 0.3 (from oblate spheroid with dielectric const $K=2$, ellipticity=0.8)

$$T = Dq^2 * 3E-8 * P(W/cm^2) * Vol(nm^3) * g$$

Oscillation, damping times for protein in laser beam.

Oscillation Period

$$\text{Energy} = -2 \rho P L^3 g q^2 / c$$

$$\text{Torque} = dE/dq = -4\rho P L^3 g q / c = k q, \quad \text{with spring constant } k$$

$$\text{Period} = 2\pi \{I/k\}^{1/2} = 13 \text{ ns. (Ribosome)} \quad \text{Moment of Inertia } I.$$

(anisotropy $a = 0.3$, 50 Watt Fiber laser, $P=2.5 \cdot 10^8 \text{ watt/cm}^2$, $L= 35 \text{ nm}$)

8ns for Lysozyme. This is small amplitude oscillation, less than 4 degrees. Doesn't matter.

Damping:

How long does it take a mol which enters at a large angle to equilibrate ? Langevin.

For Brownian galvanometer fluctuations, time to equilibrate is (Stokes damping)

$$T_d = I / (6 \pi r^3 \eta) = \mathbf{1.8 \text{ ns for Ribosome.}} \quad (\text{Viscosity } \eta \text{ for air, "independent" of pressure}).$$

Summary: Transit time 200 ns. Period 13ns. Damping 1.8 ns. **Overdamped**

Need damping gas cell near 1 atm for $l < r$, or mol in water (virus works at RT)

(For $l > r$ have effusive, Knudsen, free molecular flow. No collisions, larger T_d)

At 1 atm, $l = 100\text{nm}$. First experiments: Virus in water droplets (high damping)

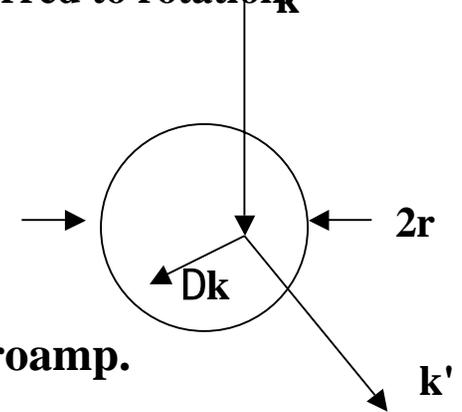
Recoil and spinning iceballs - can bragg diffraction from a levitated particle produce torque ?

Worst case, Ribosome, where all energy from "elastic" diffraction is transferred to rotation \mathbf{k}
(Translation doesn't affect diffraction much - recoil is 2 m/sec).

$$\text{Use } \mathbf{I} \omega = \mathbf{r} \times \mathbf{p} < |\mathbf{r}| |\mathbf{hDk}|$$

Find:

- *Iceball/protein starts rotating at 8×10^4 Rad/sec if free.
- *Time between electron collisions is 13 ns if beam current is 1 microamp.
- *Iceball in laser potential well **rotates by .01 deg. between beam hits. (0.06 if not bound)**
- *Transit time of mol across 10 micron electron beam is 200 ns.
- *Number of electron collisions per mol during transit is 14.
- ***X-rays**, with 5.5×10^9 phtns/sec incident, iceball rotates by $5E-5$ degrees (less p transferred)
- Consider three energies.....**
 - Potential energy of laser well at 5 degrees misalignment is 3×10^{-21} J.
 - Rotational energy imparted in (improbable) worst case (no translation) is 8.8×10^{-27} J.
 - Thermal energy at 50K is 3.4×10^{-22} J. Hence rotnl energy thermalizes, less than laser-well



Assumptions: $|Dk|_{\max} = 2 \text{ Ang}^{-1}$; Protein is Ribosome, 200kV beam, 1 microamp, 5 micron focus, LaB6. Use Langevin Brownian motion analysis - galvanometer problem (thermal, well, damping) plus e^- hits. $\Gamma = 0.6$, $T = 4K$, $P = 10^7$, $V(\text{mol beam}) = 50 \text{ m/sec}$. Inelastic Background: few phonons at 4K. $\langle \text{Torque} \rangle = \text{zero}$, in projection approx if centric or Friedel's law. **Time average torque is about beam direction** and not zero if acentric and thick (multiple scattering).

Heating effect of laser on water droplet (for virus in water).

$N = n + i k$, where $k = 3 \times 10^{-6}$ for water at one micron

Linear attenuation constant is $a = 4 \pi k / \lambda$

So $a = 12 \pi = 36$ meters for water at one micron wavelength.

Energy absorbed U produces temp rise DT where

$U = s m DT$, where DT is temp rise.

Dose due to fluence I results in energy absorbed

$U = a * I * V$ where I is incident flux in watts/m²/sec, V is volume

So $DT = a * I * V / (s m) = a * I / (s * r)$ per second

where $r = 10^3$ Kgm/m³ is density of water, $s = 4.2 \times 10^3$ J/Kgm/K is specific heat of water.

So,

$DT = 3.6 \text{ K in } 200 \text{ nanosec.}$

for a one micron cube of water illuminated by

2×10^8 watts per cm² of laser light at one micron wavelength. Independent of mass.

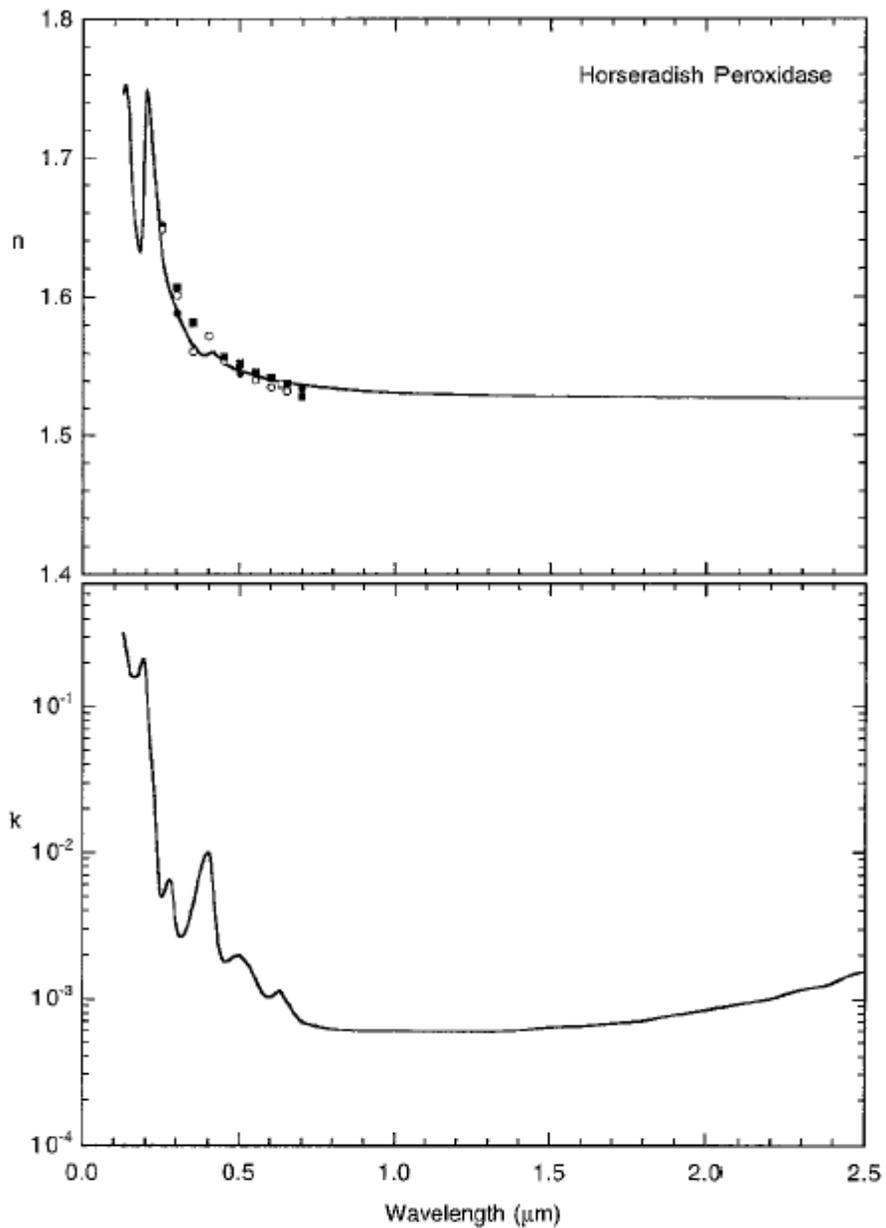


Figure 4. Optical constants of horseradish peroxidase from 0.13 to 2.5 μm .

E. Arakawa et al (1997) 308 amino acids +

•No peaks around one micron at RT.

•Temp rise due to non-resonant optical absorption ?

Compare to femtosecond XRD. (Neutze et al., Nature 2000)

- i) We avoid the need for femtosecond pulsing. (cf LCLS at SLAC, Hadju/Neutze scheme)
- ii) We avoid the need to find the orientation relationship between patterns from successive randomly oriented mols.
- iv) No radiation damage issues - transit time allows much less than critical dose.

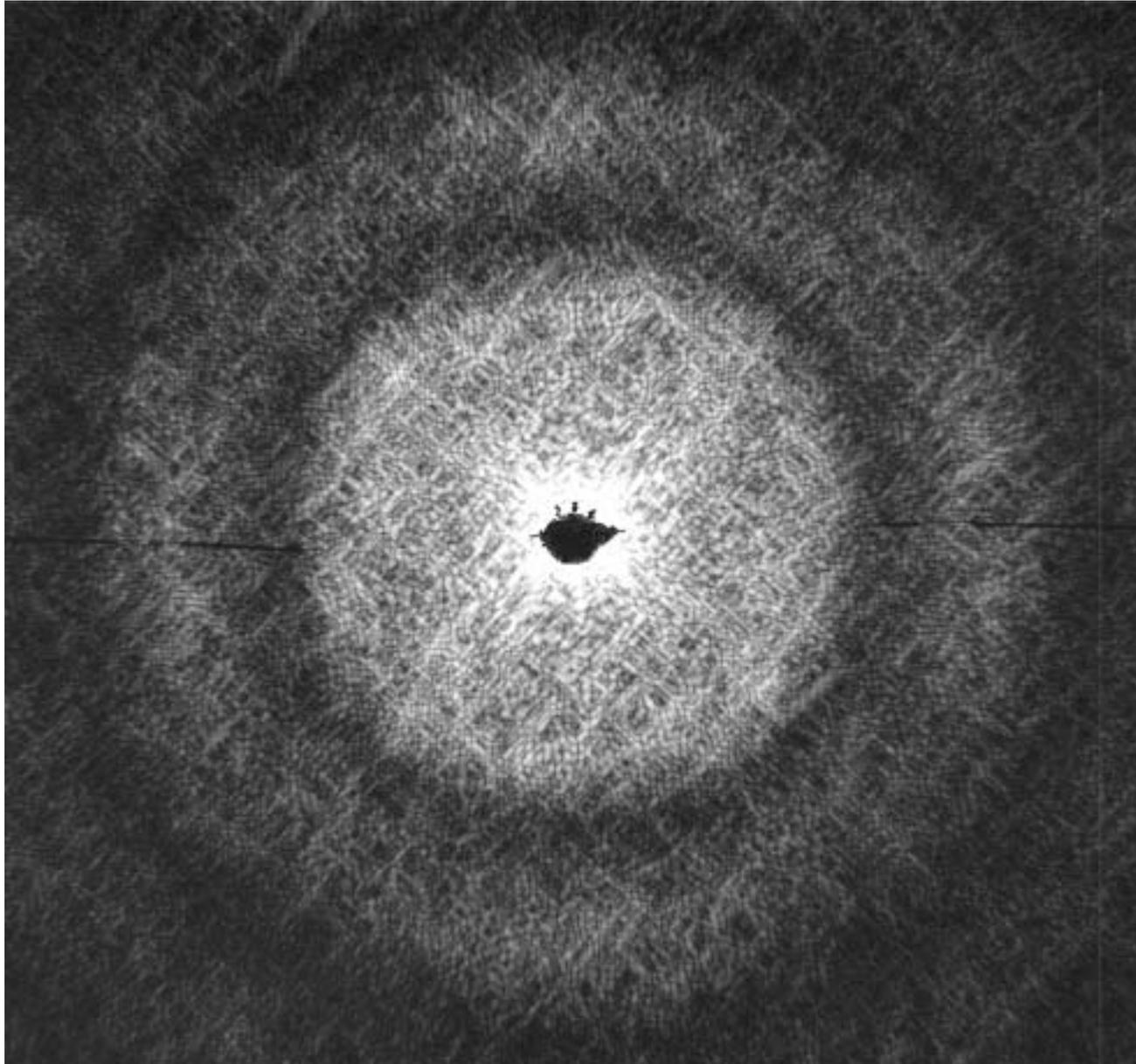
OR:

Use femtosecond X-ray pulses on a swarm of laser-aligned atoms for time-resolved diffraction with more signal. (H. Chapman).

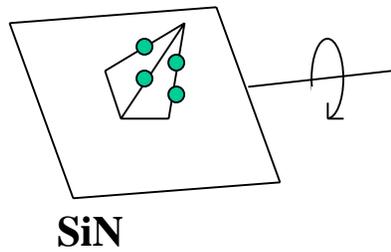
The phase problem is solved by iterative Fienup-Gerchberg-Saxton methods plus heavy atom.

How to solve the phase problem by iterative methods

600 eV soft X-ray transmission pattern from 50nm gold ball clusters



- 130 views: $\pm 65^\circ$ in 1° steps
- Exposures of 3, 20, and 200 seconds at each angle
- Automation is now developed to where we can take this largely unattended
- Total data collection time about 10 hours
- First 3D data set taken with the Stony-Brook-Brookhaven chamber
- Speckles are well developed and often round i. e. not radially streaked which shows that the system bandwidth is now good
- Note the envelope of the pattern is the Airy disk of one ball
- Midpoints of the sides correspond to a spatial period of 17.4 nm or a Rayleigh resolution of 8.7 nm



**A.Barty, H. Chapman, S. Marchesinini, U. Weierstall, H. He, C. Cui, M. Howells, J. Spence. ALS 04
Unpublished.**

This is a 3D movie.

First atomic-resolution diffractive image reconstruction.

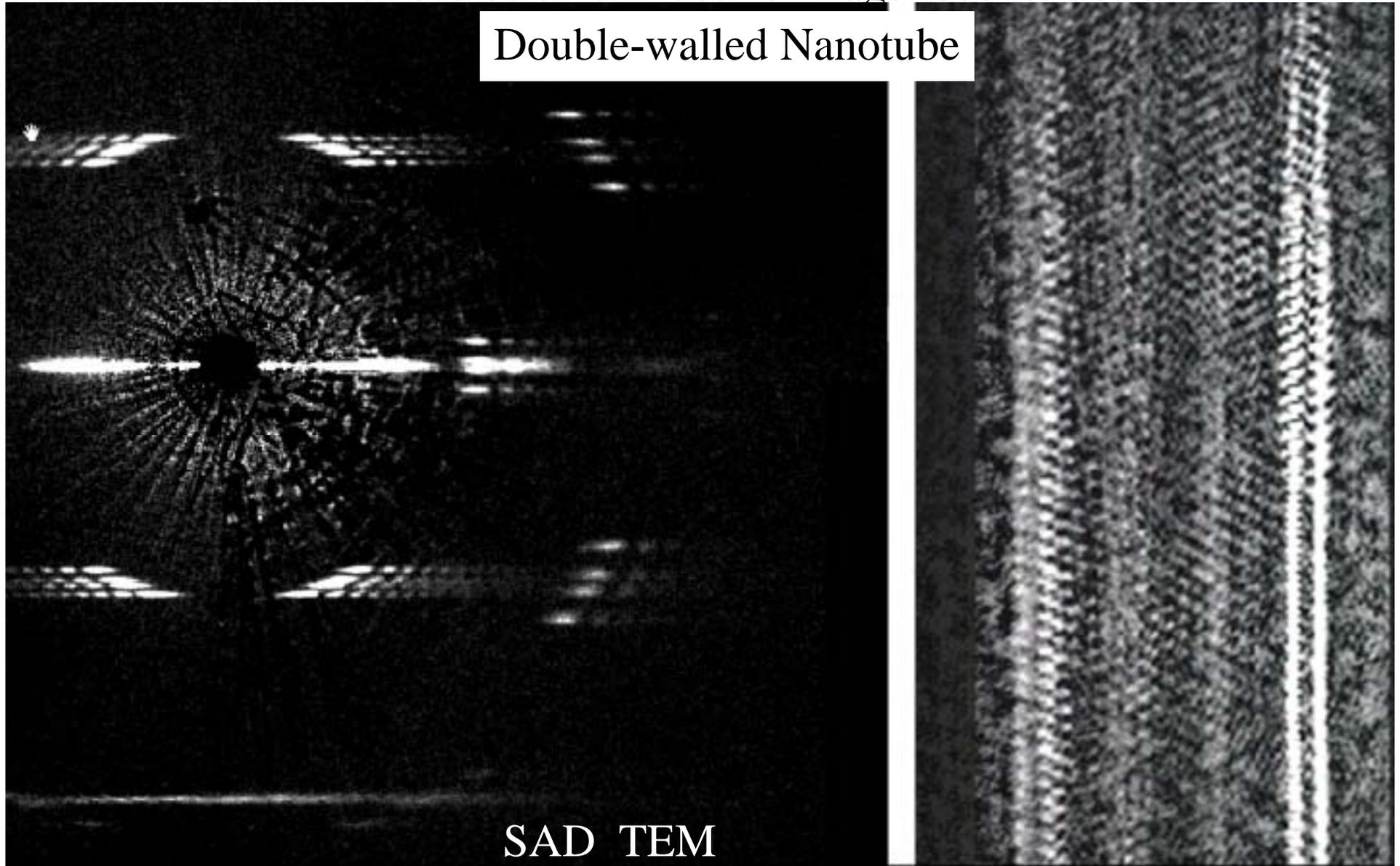


Image reconstructed from electron-diffraction pattern by HiO
Requires image plate, not CCD. Exposure time 12 secs. **J.M.Zuo et al Science 300, 1420 (2003).**

Simple calculation of exposure time for electron diffraction (scaled to nanotube experiment).

The 7 mols within the beam are constantly replenished, to avoid damage.

(each receives less than critical dose (at atomic resolution) during transit)

Hence exposure time is the same as for 7 stationary molecules.

Exposure time for Zuo's TED pattern from one double-walled nanotube was 12 secs.
Length is about 20nm.

scattered particles per second = number incident per sec per area * number of mols * cross section

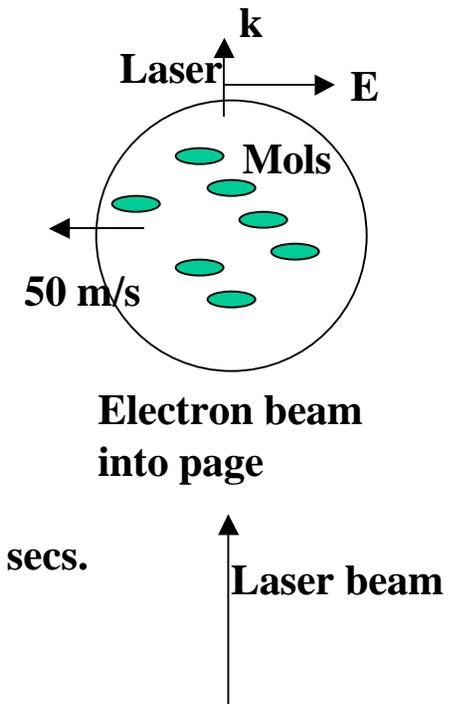
Hence #scattered is propn to incident flux and number of mols.

The LaB6 source we will use is 100 times less bright than the Field-emitter Zuo used.
Assume n mols within 5 micron aperture, each same size as 20nm nanotube.

Then exposure time is $(12 * 100/n)$ secs. For single-file 50nm iceballs, $n = 40$, so time is **30 secs**

Note: Protein has much higher atomic density than nanotube, hence this is upper limit).

(Compare with estimate of 80 secs for closely spaced much bigger 0.5 micron iceballs).

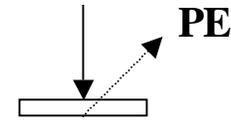


Compare exposure times for electron diffraction and ALS.

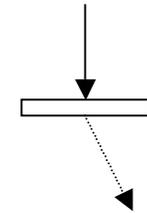
Electrons

XRD

Background "No"background
More cps(source,S) Less cps
Mult Scat No Mult Scatt. Easy quantification
Coherence OK for both, time and space.



XRD
no background



TED
background

Exposure time for ALS. Assume present flux of BL 9.0.1 , $E/DE = 500$, ZP mono.
 $S = 665 \text{ Ang}^{-2}$ for Ribosome (35nm long). Take 50nm diam iceball, ($D=0.026$)

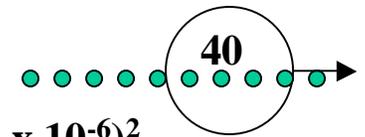
$$S_{in} = 3 S_{el}$$

$$q_E = DE/2E$$

$$\# \text{ Xrays/sec} = (\# \text{ incident/sec, given area}) * (\# \text{ of mols in this area} * S) / \text{area.}$$

$$= 5.5 \times 10^9 * 40 * 665 \times 10^{-20} / (4 \times 10^{-6})^2$$

$$= 91438 \text{ cps, or } 0.1 \text{ cps per pixel on } 1 \text{ K}^2 \text{ CCD.} \quad (\text{Current cocolith gives } 8)$$



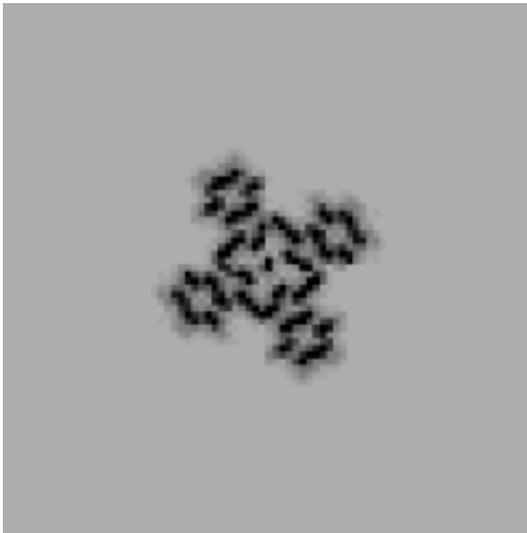
Conclude: About **80 secs per orientation using electron diffraction**; **2.5 hrs gives 1000 cpp. for XRD**
 Need present XRD flux at 6 kV (2 Ang) to get near-atomic resolution tomography of proteins.

Conclude: Need 3D spray source for XRD. Then $40 \rightarrow 40^3$, get **6400 cps per pixel for XRD.**

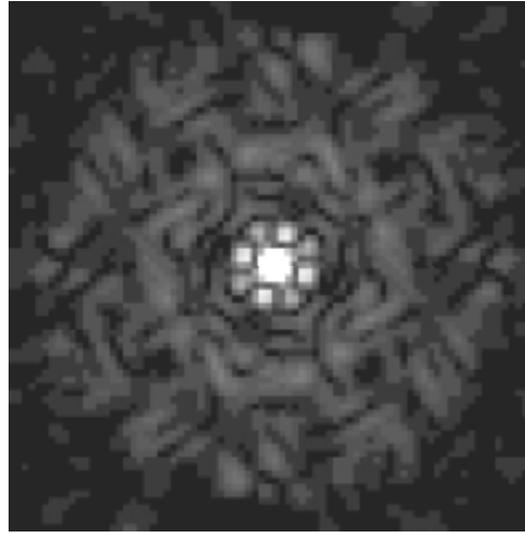


Two-dimensional simulations for effect of partial alignment on HiO reconstruction

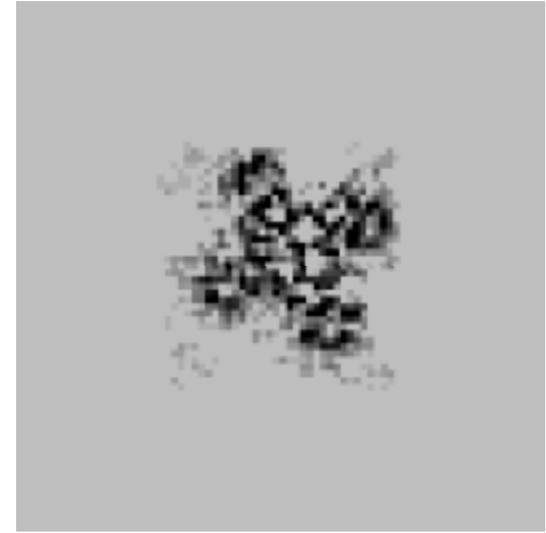
Simulated projected potential for CuPC



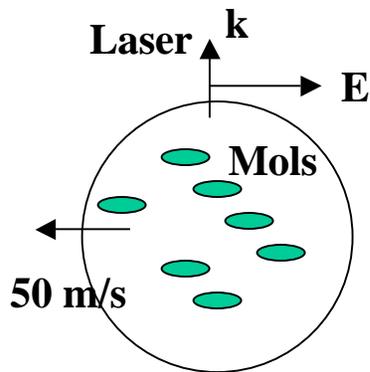
Sum of 100 diffraction intensities with 5 degree wide normal distribution



Reconstructed image by HIO



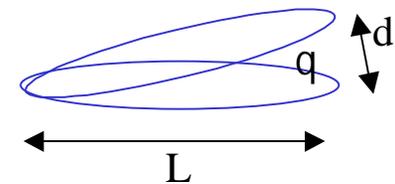
Copper Phthalocyanine molecule



Electron beam into page

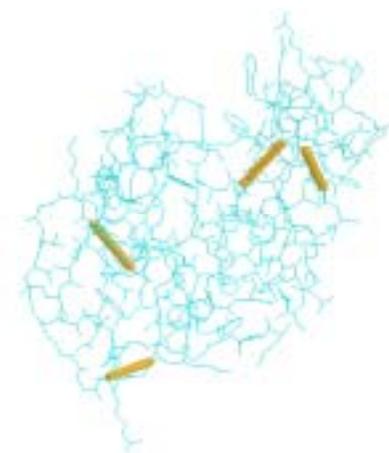
Partially aligned diffraction patterns added together.

Reconstructed image

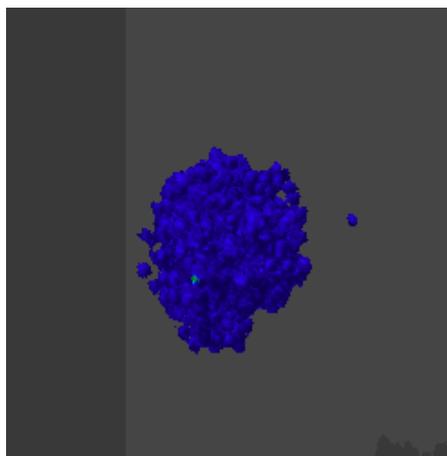


The resolution limit is $d = L q_{\text{rms}} = 1 \text{ nm}$.

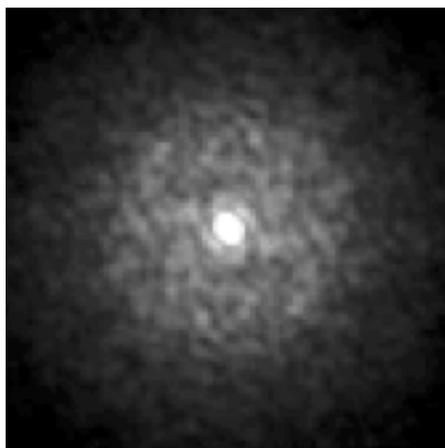
Simulations for one Lysozyme molecule 5LYZ.



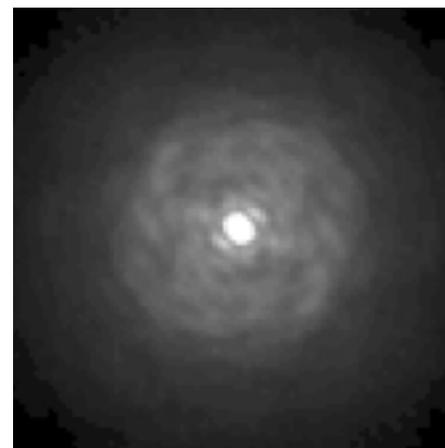
MDL



Electrostatic potential



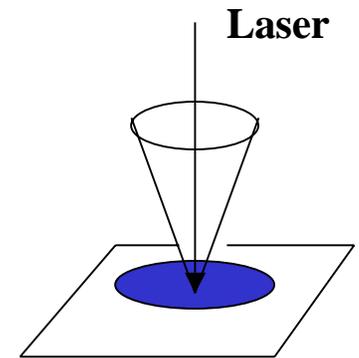
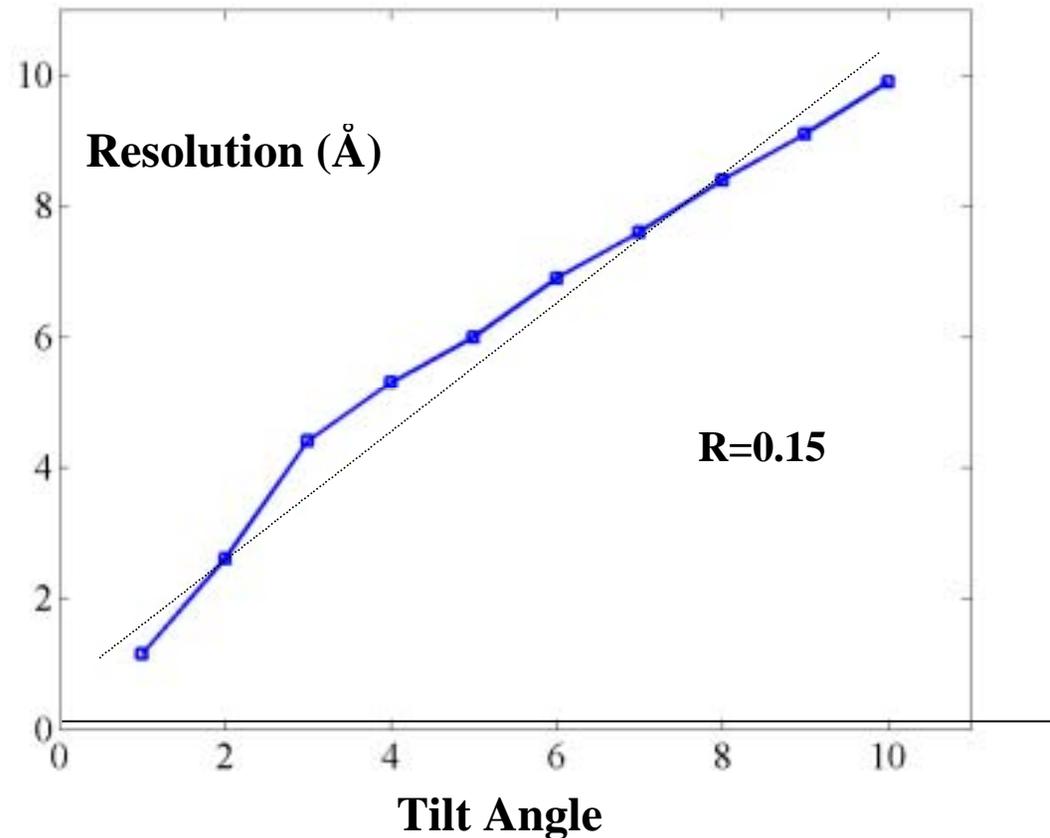
Electron diffn pattern.



**TED averaged over 5 degree solid angle
(cone angle about c axis. 0.02 str).**

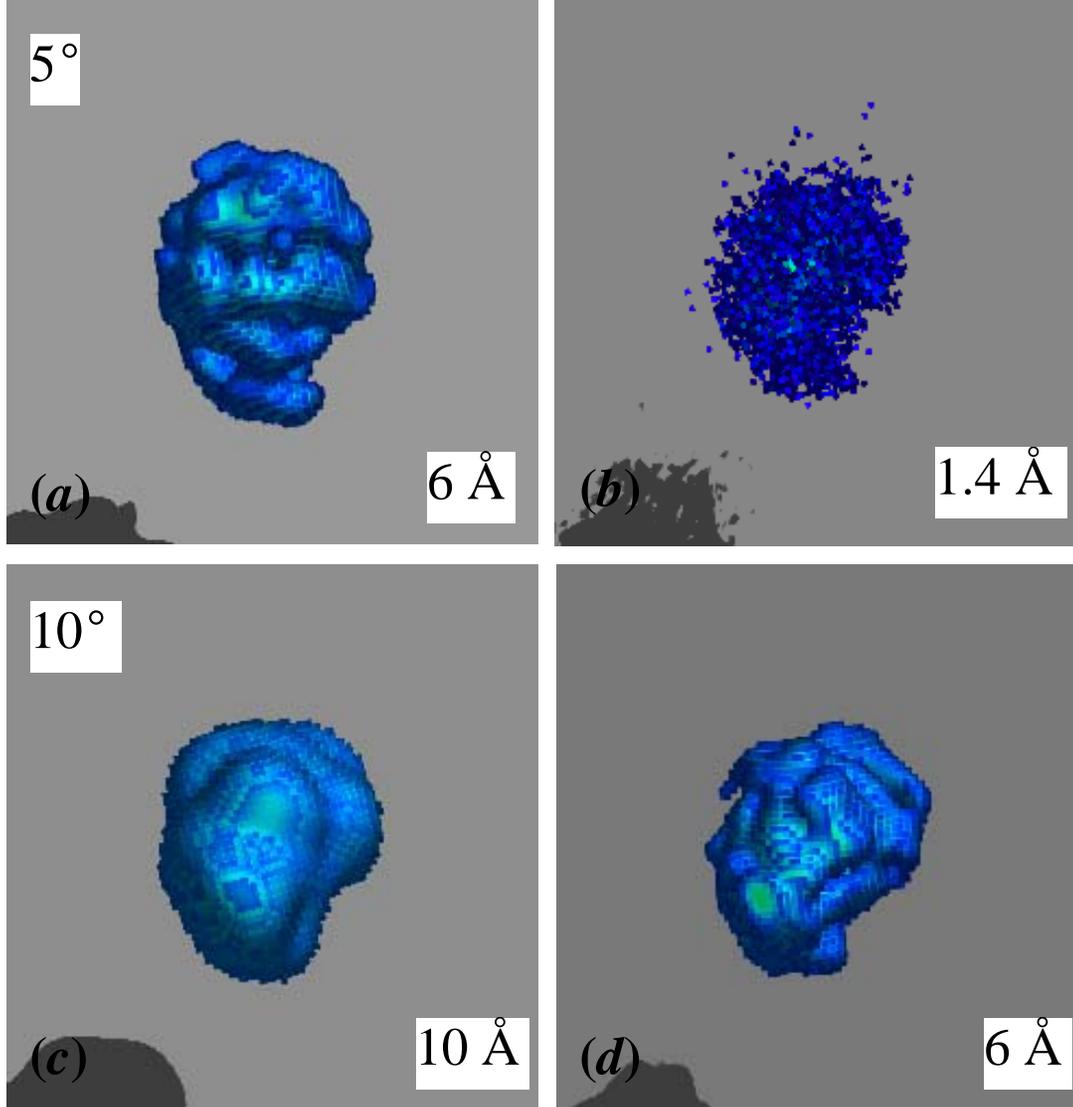
8008/4 non-H atoms from PDB. Cell dimensions 9.5 X 9.5 X 45 nm.

Plot R against resolution for several misalignment angles. Chose R values at R = 0.15 for good image. (High resolution with poor R factor gives unfaithful reconstruction).



**Resolution against misalignment angle for 5LYZ
with R-factor held at R= 0.15 for faithful reconstruction.
Best fit occurs for $R = Dq L$ with $L=4.5\text{nm}$
5 degrees cone angle misalignment and 0.6nm resolution is possible.**

3D reconstructions using HIO for 5LYZ.



Diffraction patterns had 5 or 10 degree misalignment. (cone angle)

R-value is held at 0.15.

Only 5° misaligned at 6 Å res. is useful. Higher res detail misleading.

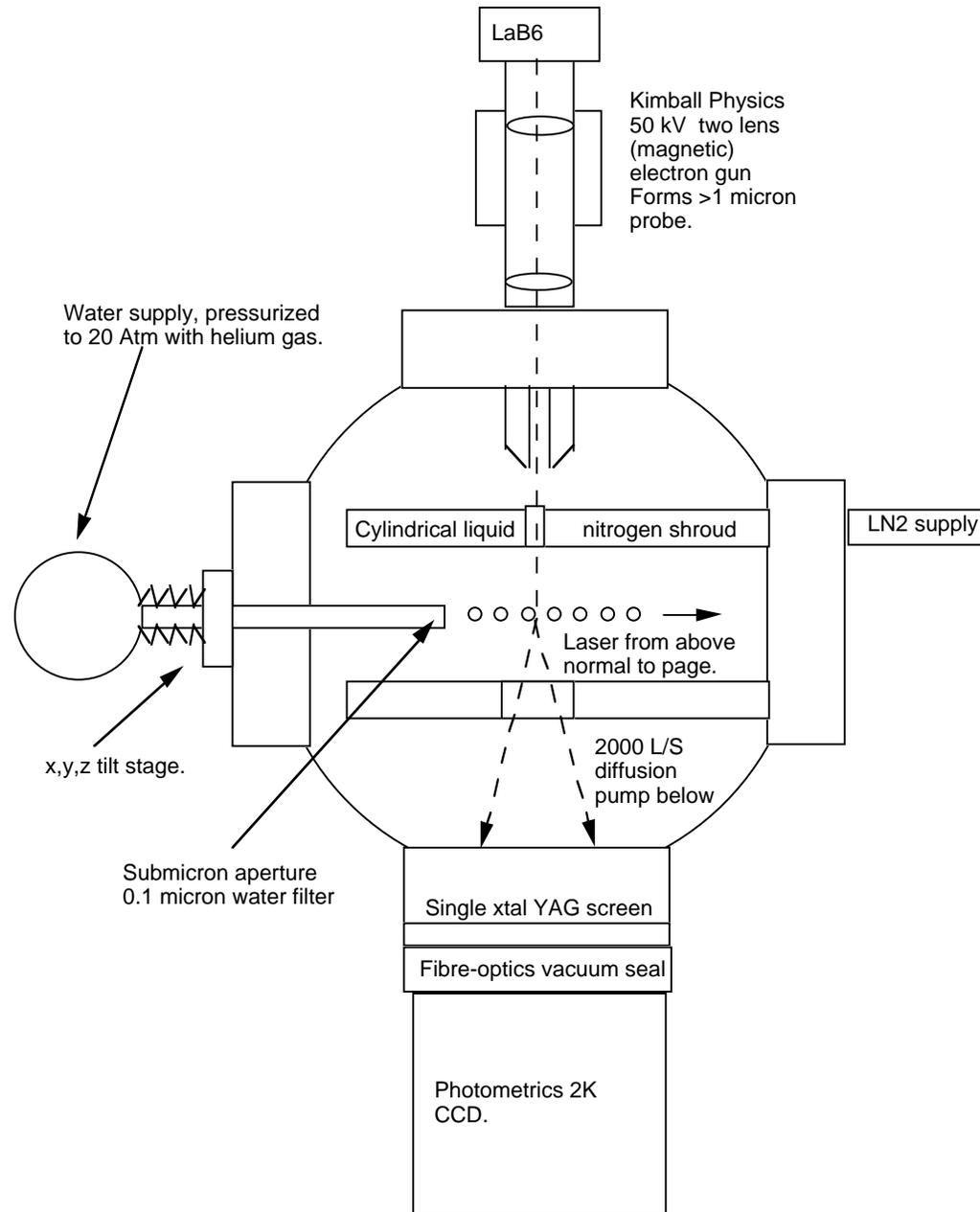
Conclude: Secondary structure can be seen with 5° misalignment at 0.6nm resolution.

This requires $T = 2 \text{ K}$ and $P = 10^8 \text{ W/cm}^2$ (max CW P for tuneable Ti:Sapp) if our anisotropy factor is correct.

Increase $D\alpha$ near resonance ? (higher temp)

Much more power from fiber laser, hence higher T.

General arrangement
of Serial Diffraction
apparatus in Spence Lab.
for water/mol jet.

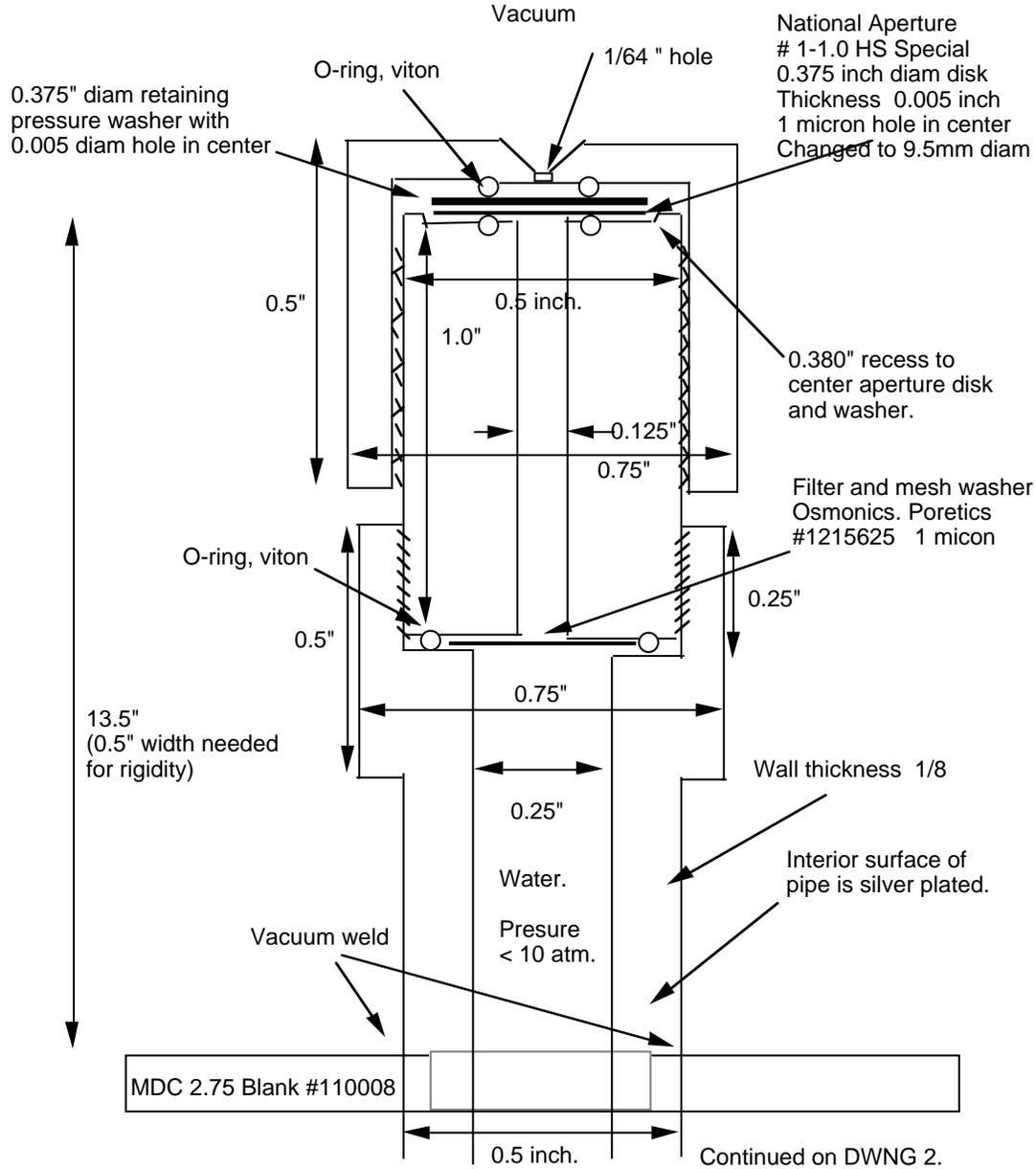


Water jet for electron diffraction
DWNG 1

J.Spence Feb 04.

Thickness of z motion 3" (flange to flange fully compressed)
 Thickness of filter 2 5/8".
 Total length of pipe = 6" + 1 7/8" + 2 5/8" + 3" = 13.5"

Some dimensions not to scale.

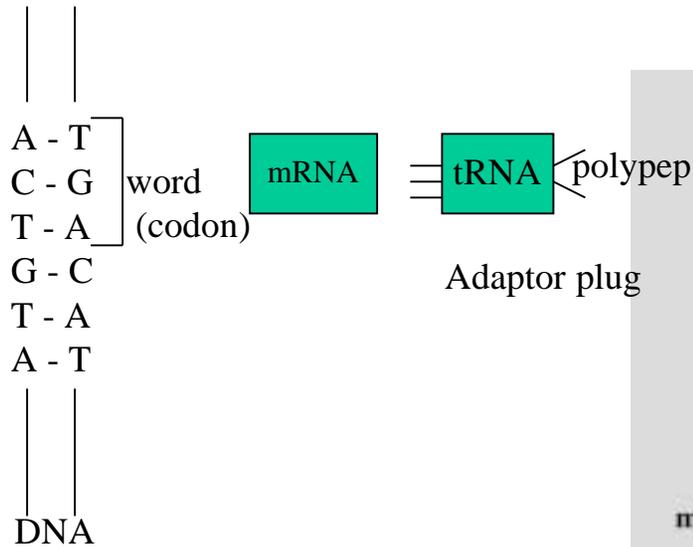


Serial Crystallography. Summary.

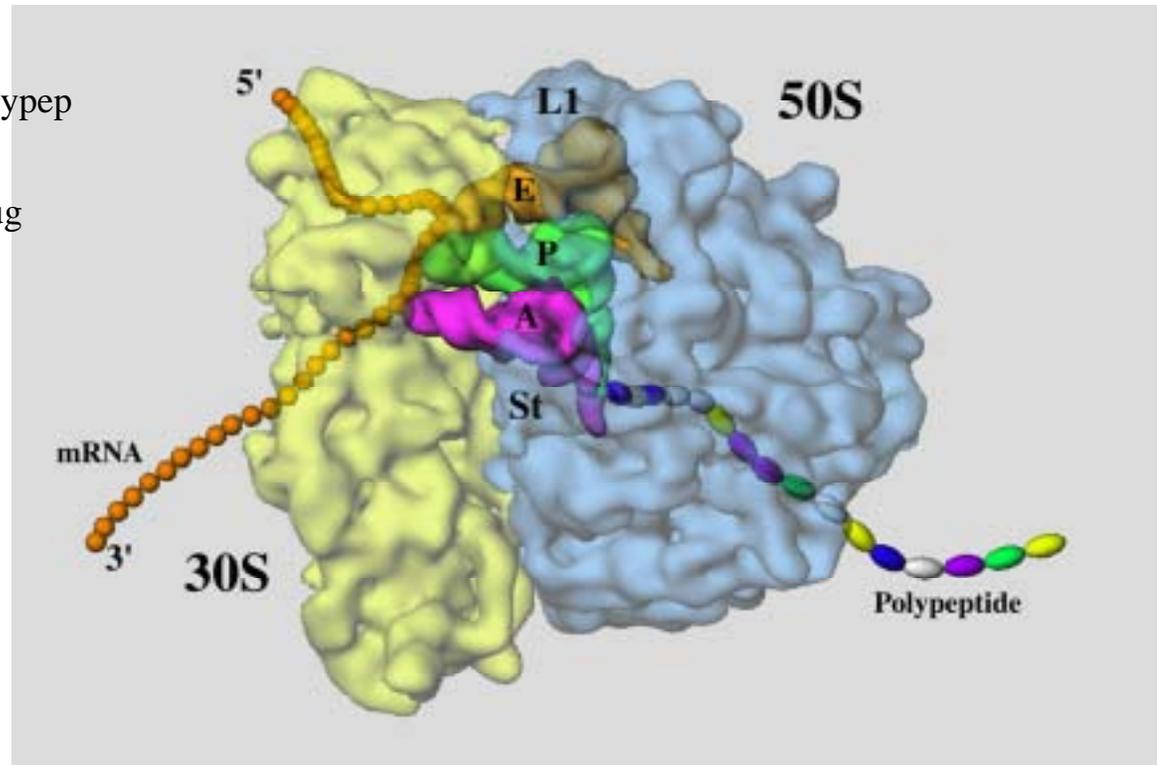
1. Three-dim orientation of small organic mols has been demonstrated in molecular beams.
2. Electron diffraction from molecular beams has been demonstrated.
3. Formation of superfluid submicron liquid helium droplet beams has been demonstrated
4. Optical spectroscopy of amino acids has been demonstrated in helium droplets at 0.4K.
5. Electron diffraction from xtalline ice droplet beams has been demonstrated. (Bartell)
6. Electrojet-based mass spectroscopy is mature.
7. Electron sources are brighter than synchrotrons, and elastic e⁻ scattering is 10⁵ times greater than X-rays with less damage per unit elastic scattering. But no ms with X-rays, better quantification, less background (no "inelastic scattering"). Damage with Xrays is greater for imaging, less for spectroscopy.
8. Solution of the phase problem has been demonstrated for non-periodic real objects using both electron and X-ray scattering data.

The competition - tomographic imaging of proteins at 1nm resolution by cryo-EM

Protein synthesis (“Life itself”) in the Ribosome: The ribosome structure determined to 1nm resolution by TEM (tomographic cryomicroscopy). J.Frank et al.



- 4 nucleic acids
- 2 (4) base pairs
- mRNA reads one side only
- 3 pairs per word (per amino)
- $4^3 = 64$ possibilities per amino
- 20 amino acids.
- n words per gene (protein)



Ribosome width 25nm

(Cell,100, p.537 (2000))

Experimental e-coli ribosome reconstruction from TEM images of non-crystallised mols in ice. mRNA bring 3-bit codons from DNA. tRNA “adaptors” (E,P,A) have plugs at one end to mRNA codon, at the other to an amino acid, which is added to the polypeptide chain as the ribo runs along the mRNA. Chain will fold to become a new protein. (also Baumeister).

This is a movie

Tomography in TEM. Mesoporous silicate catalyst with Pt particle.
Hole diameter 2.5nm. Resolution 1nm P Miegley, Cambridge UK.

Summary. Serial Crystallography.

1. Existing CW fiber lasers should align large (eg ribosome, 35nm) proteins in a beam to within a few degrees at any temp below -40 C.
2. The Feinup HIO algorithm and variants can perform the inversion of the diffraction patterns from such a beam to a tomographic charge density.
3. Misalignment in the diffraction data of a few degrees results in faithful tomographic images at a resolution of about 0.6nm, at which secondary structure (alpha-helices, beta sheets) can be seen.
4. Experimental ED work is starting in Spence lab at ASU.
5. Experimental XRD at ALS would be possible at 10nm resolution now. At atomic resolution after upgrade to 6 kV. Recording times reasonable if "shower head" droplet source used, 50nm holes.
6. Compare with other methods (NMR, cryo-EM): smaller mols than cryo-EM, higher res than cryo-em., Larger mols than NMR. Higher throughput. Limit conformational variability by adding small mol ? (cf cryo-em).

Details: Spence, Doak. PRL 92, 198102 (2004) and Spence et al Acta A, 04 in press.