Technical challenges

NRAMM Workshop

Scripps - 8th Nov 2009

Richard Henderson

State of the field

- Some excellent 2D crystal structures
- Some very good structures from helical arrays
- Some impressive icosahedral structures, making use of symmetry
- Good single particle structures without symmetry
- Progress with resolving multiple states
- Awareness of need for quality control indices
- Electron tomography making increased impact

Technical challenges to progress

- Prerequisite is homogeneous well-preserved specimens
    - blotting
    - cryosectioning
    - surface forces
- Signal-to-noise ratio in images
    - B-factor - describes fading of contrast with resolution
    - Radiation damage - unavoidable
    - Charging
    - Movement
    - Contamination
- Quality control indices
- Detectors need higher DQE
- Automation
- Computer programs (parallelisation, graphics chips)

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Human Rotavirus DLP
3.8 Å, B-factor 450Å^2

Zhang et al & Grigorieff (2008) PNAS **105**, 1867-72.

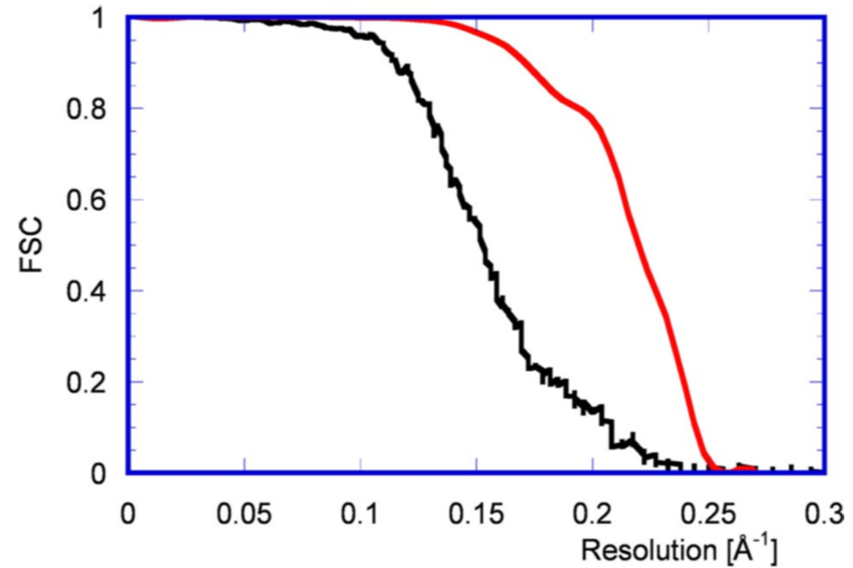


Fig. 4. FSC curves before (black) and after (red) 13-fold nonicosahedral averaging. The black curve suggests a resolution of 5.1 Å (0.143 threshold value), and the red curve indicates a resolution of 4.1 Å.

a X-ray

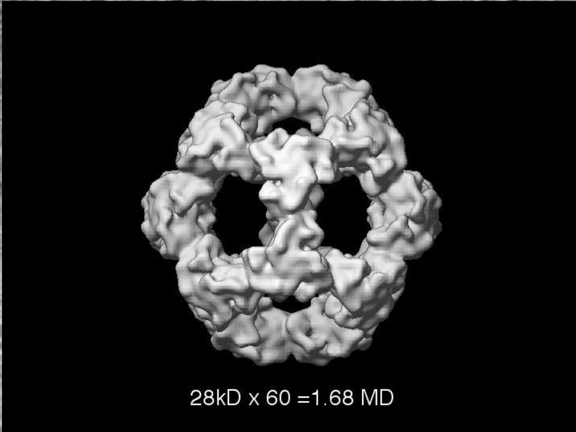
b cryoEM
Lys125
Ile122
Asn128
Phe129
Ile127
Phe124

c
Ser186
Arg 325
Glu 187
Tyr 160
Trp 181
Leu 322
Thr 179
Phe 164

Rosenthal & Henderson (2003) - three main points

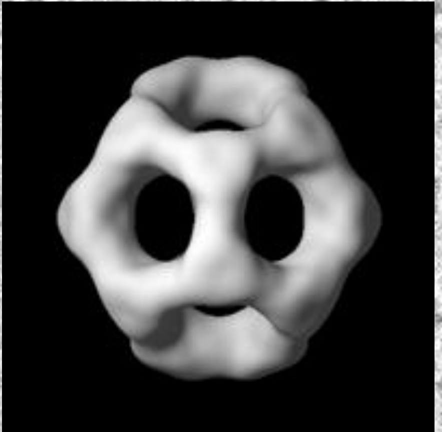
- More realistic (less conservative) resolution criterion (FSC = 0.14)
- Sharpening map and f.o.m. weighting
- Tilt pair validation of orientation angle determination

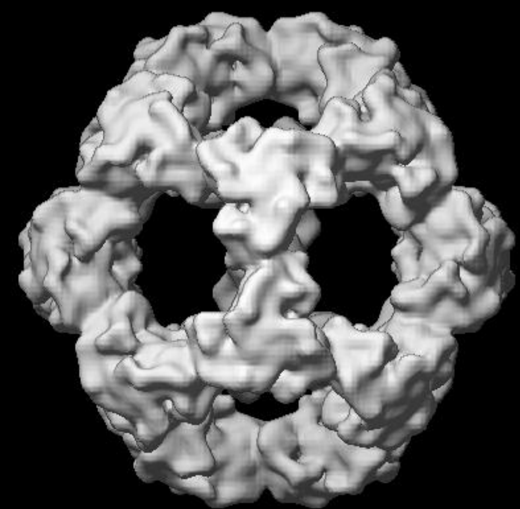
2

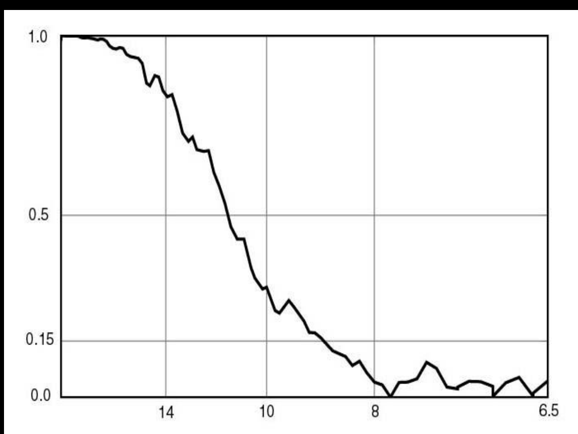


3

5

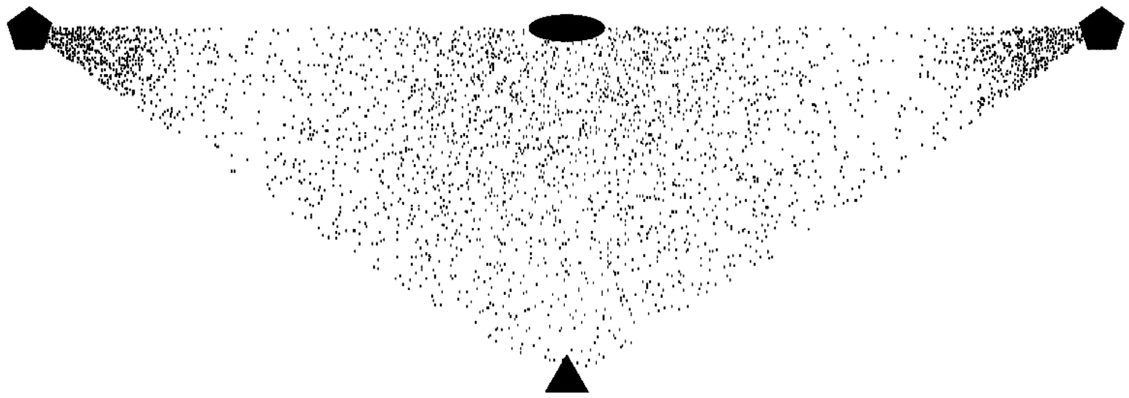






1.0
0.5
0.15
0.0
14
10
8
6.5

Particle distribution



Fourier shell correlations

$$C_{ref} = (2*FSC/(1+FSC))^{0.5}$$

1.0

0.5

0.143

C

C_{ref}

C_{XRAY}

FSC

14

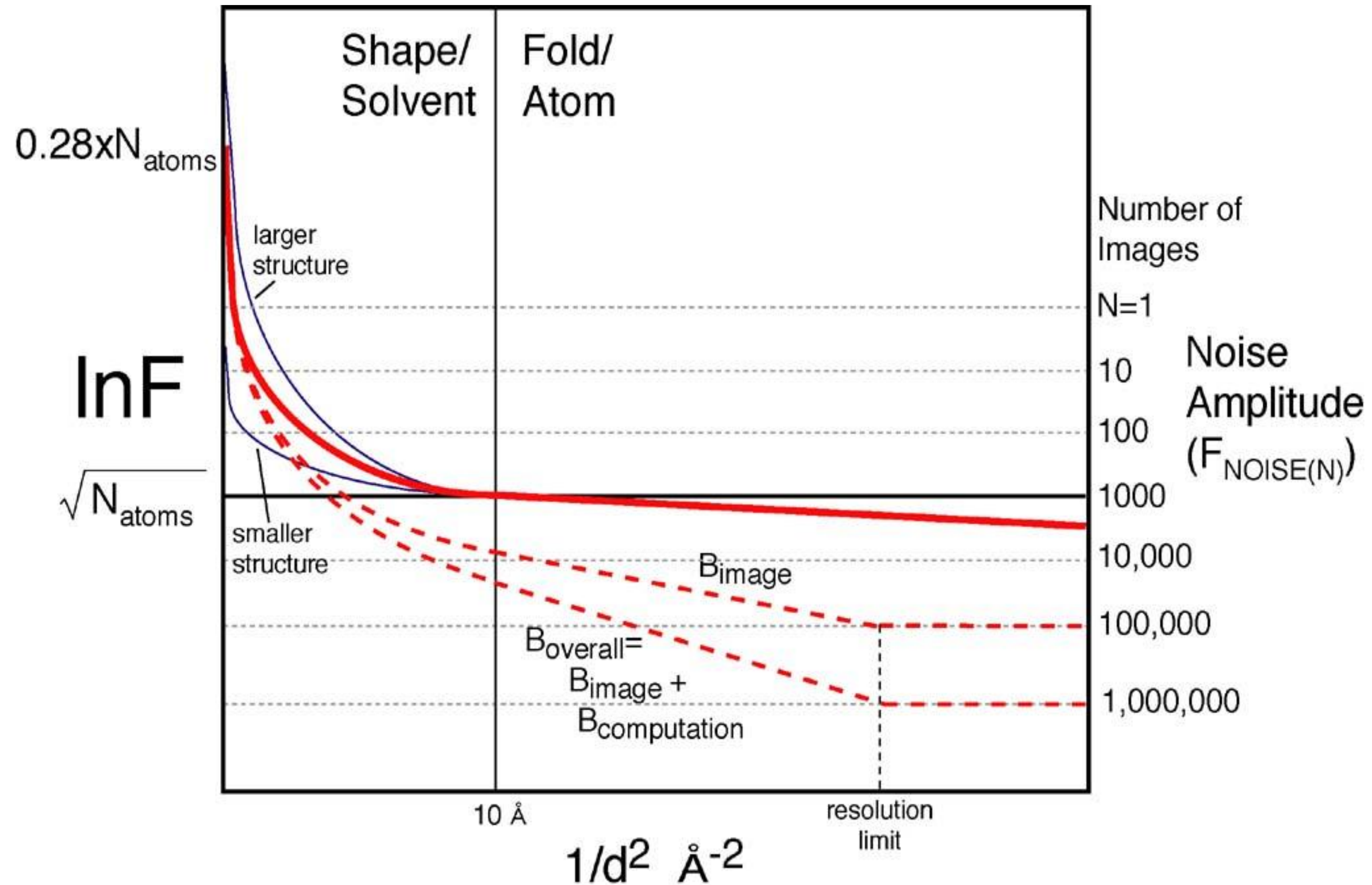
11

8.7

6.5

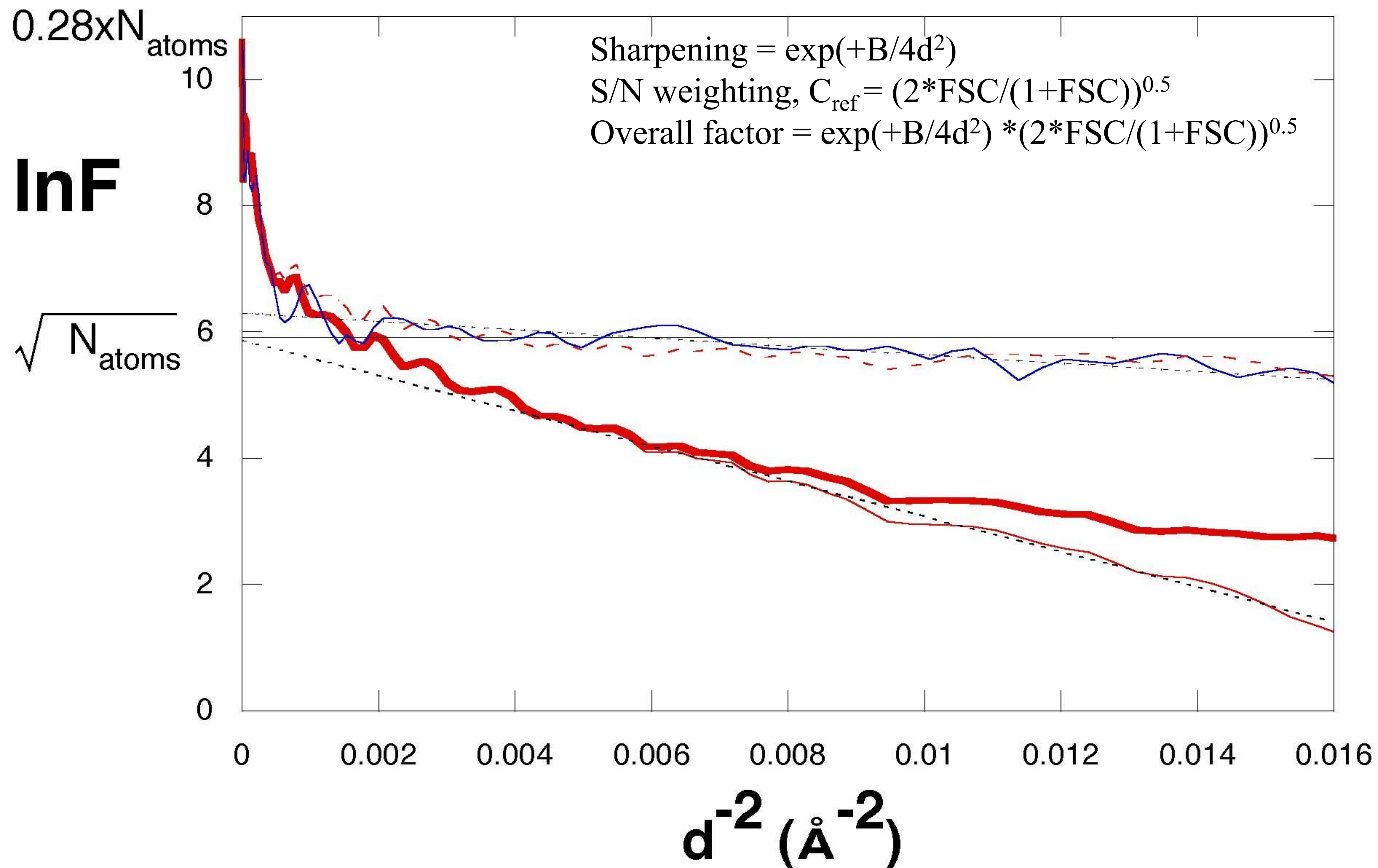
d^{-2} (Å)

Theory – single particles in ice



Experimental data

Rosenthal (2003) JMB **333**, 225-36
Fernandez (2008) JSB **164**, 170-5



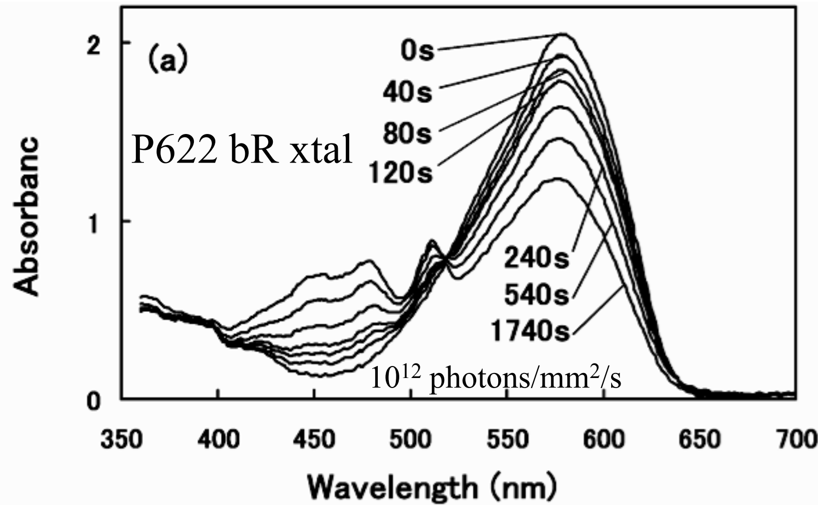
Radiation damage in structural biology

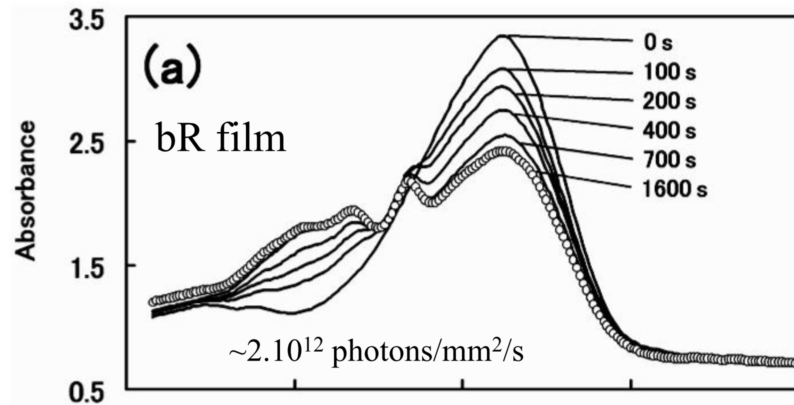
- Three-dimensional crystals (X-ray) contain ~10^{10} molecules
- Two-dimensional crystals (EM) contain ~10^4 molecules
- Single particles contain 1 or a small number of copies

- Radiation damage unfortunately makes it impossible to determine the structure, except at > 2-4 nm resolution, without some averaging
- Current challenge is to understand how much averaging is necessary in theory and to try to get close to this in practice

Matsui .. & Kouyama (2002) JMB 324, 469-81

Damage induced by X-irradiation of bacteriorhodopsin





(a) SB D85 D212 R82 E204 E194
(b)
(c)
(d)

Doses = 4, 8, 12, $16{*}10^{15}$ photons/mm^2

bR in crystals or membranes show similar sensitivity to irradiation

10^{16} photons/mm^2 => 5 el/Å^2 = normal cryo-EM exposure - carboxyl groups fall off

$4{*}10^{15}$ photons/mm^2 => 2 el/Å^2 = dose/frame in above X-ray sequence

$2{*}10^{14}$ photons/mm^2 => 0.1 el/Å^2 = safe dose where no damage of any kind is detectable

Unwin & Henderson (1975) JMB

Stark, Zemlin & Boettcher (1996) Ultramicroscopy

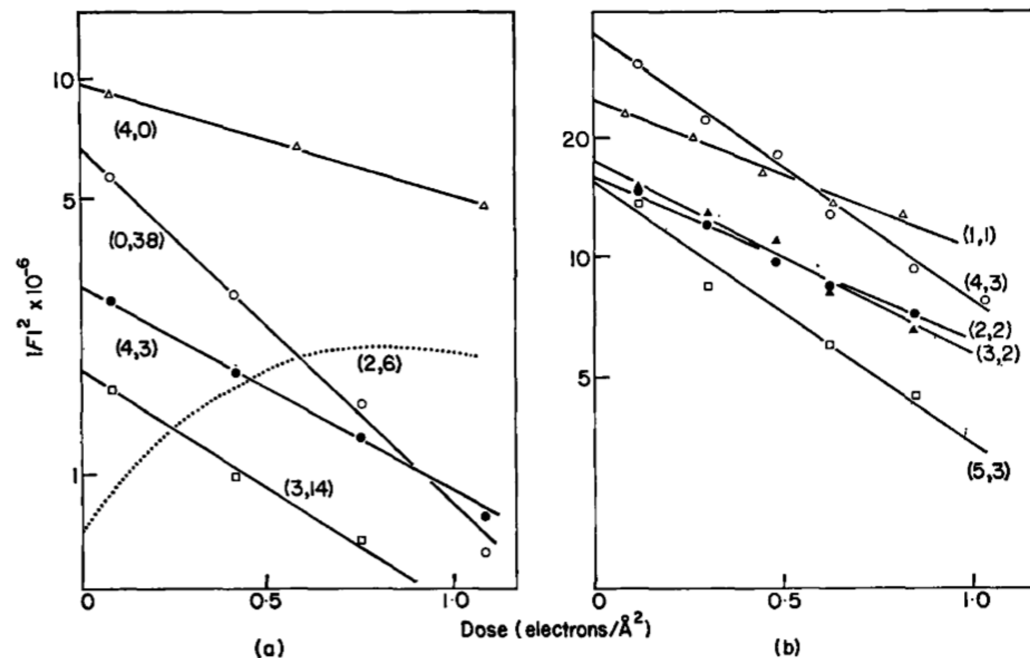
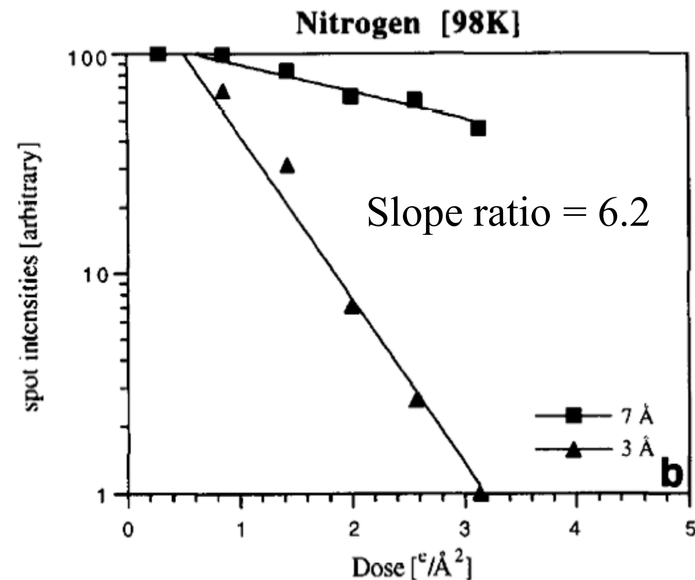
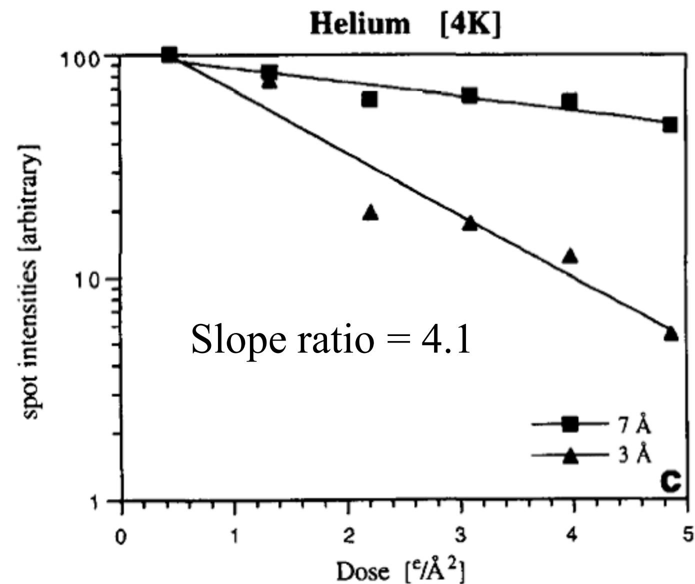


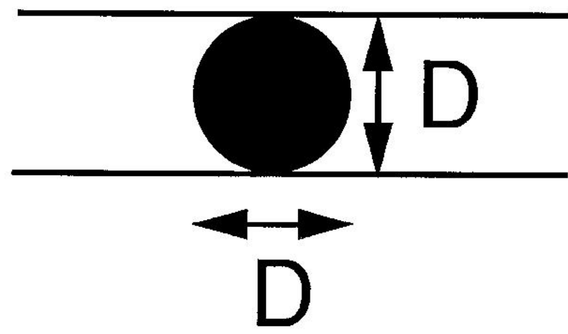
FIG. 1. The intensities (on a logarithmic scale) of some typical reflections in (a) the catalase and (b) the purple membrane electron diffraction pattern, plotted as a function of electron dose.





Conclusions

- 3Å data is more radiation sensitive than 7Å data by a factor of 4.1x to 6.2x.
- This translates into a B-factor due to radiation damage of B = 90Å^2 at 98K, or B = 70Å^2 at 4K


D
D

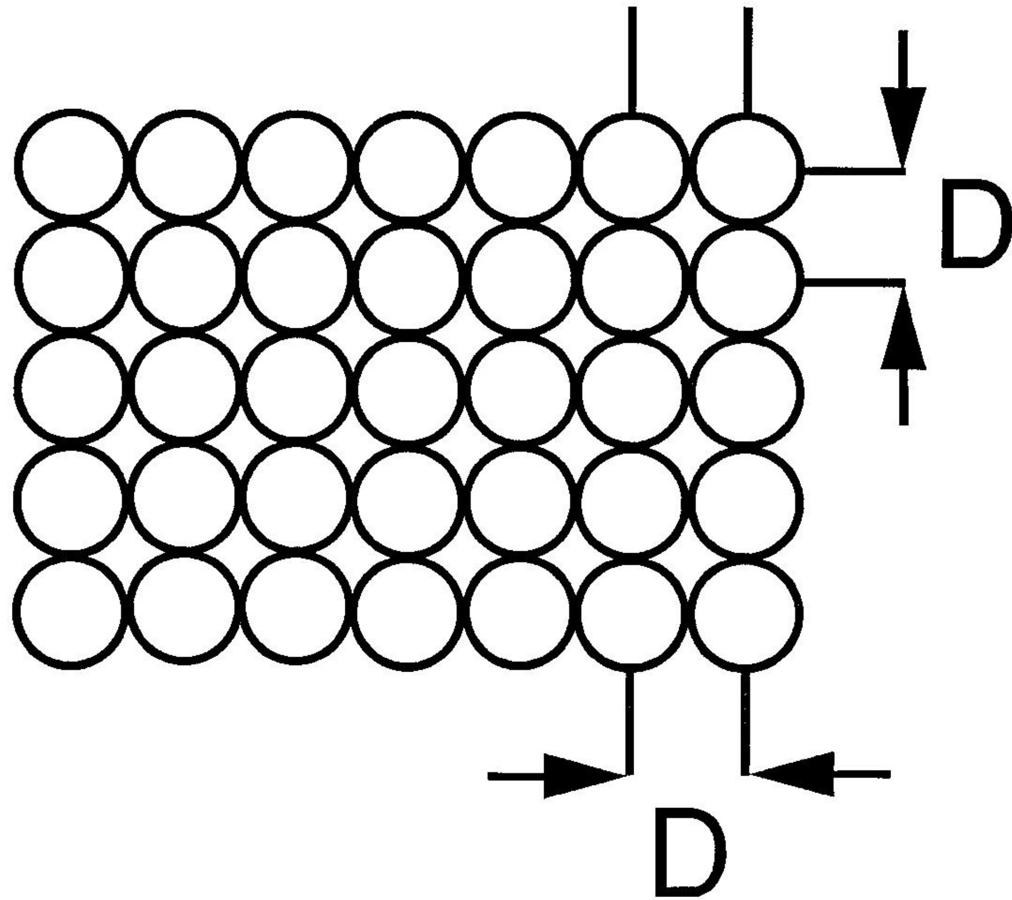
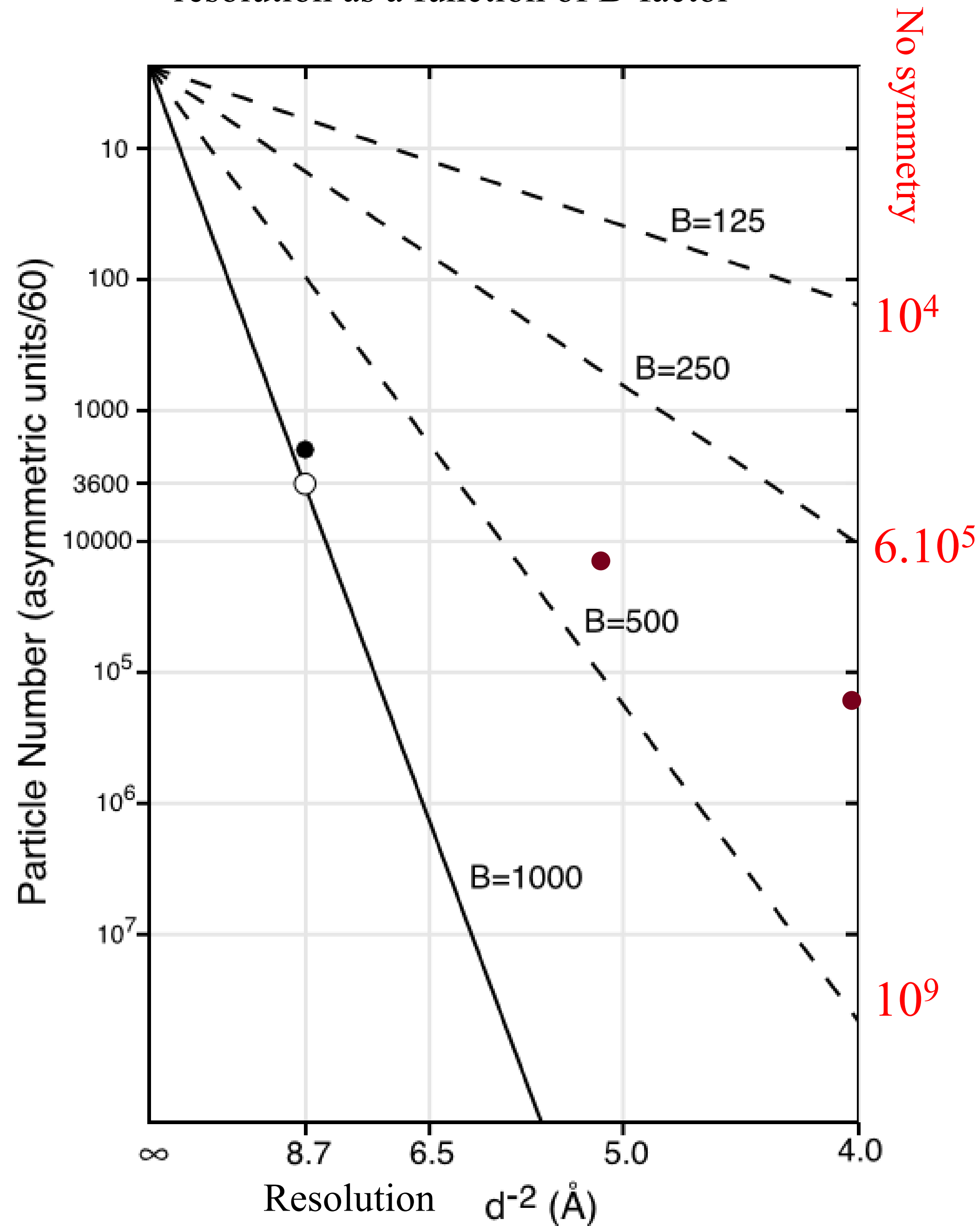

D
D

TABLE 2

Type of molecule	Approx. M.W. (Daltons)	D (Å)	N_c, number of carbon atom equivalents	N_s, number of unique diffraction spots to resolution of d = 3Å in projection	f, fraction of electrons elastically scattered out to 3Å resolution	$\frac{\langle I_{OBS}\rangle}{I_o}$	$\frac{\langle F_{OBS}\rangle}{F_o}$	Phase contrast = total image fractional contrast = signal	Fractional noise level in pixel of dimension $\left(\frac{d}{2}\right)^2$ = 1.5Å x 1.5Å	Can single molecule be detected? How many times > noise	Multiple of sigma expected within unit cell at random	Multiple of sigma expected within entire volume of 5 parameter space at random	Can single molecule alignment be carried out in practice?	Minimum number of images needed for structure with average Fourier component to be >3 σ in projection	Total number of images in 3D x $[\frac{\pi D}{d}]$ De Rosier & Klug (1967)
large virus	300M	900	25,000,000	141,371	0.0520	0.184×10^{-6}	0.429×10^{-3}	0.322	0.30	644	5.2	8.5	yes	13	12600
small virus	11M	300	936,000	15,707	0.0173	0.552×10^{-6}	0.743×10^{-3}	0.186	0.30	124	4.8	7.7	yes	40	12600
ribosome	3.3M	200	277,000	6,981	0.0115	0.827×10^{-6}	0.910×10^{-3}	0.152	0.30	68	4.7	7.5	yes	60	12600
	1.4M	150	117,000	3,926	0.0087	1.103×10^{-6}	1.050×10^{-3}	0.132	0.30	44	4.6	7.3	yes	80	12600
multimeric enzyme	420K	100	35,000	1,745	0.0058	1.654×10^{-6}	1.286×10^{-3}	0.107	0.30	24	4.4	7.1	possibly	120	12600
	180K	75	14,600	981	0.0043	2.206×10^{-6}	1.485×10^{-3}	0.093	0.30	16	4.2	6.8	possibly	160	12600
	52K	50	4,330	436	0.0029	3.309×10^{-6}	1.819×10^{-3}	0.076	0.30	8.4	4.1	6.7	possibly	240	12600
small protein	18K	35	1,500	213	0.0020	4.727×10^{-6}	2.174×10^{-3}	0.064	0.30	4.9	3.9	6.3	no	345	12600
very small protein	7K	25	540	109	0.00144	6.618×10^{-6}	2.572×10^{-3}	0.054	0.30	3.0	3.5	5.9	no	480	12600
equation	(1)	-	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(13)	(16)	(17)	(10)	(11)
relation to D	$0.418 \times D^3$	D	$0.0346 \times D^3$	$0.01745 \times D^2$	$5.7 \times 10^{-5} \times D$	$1.654 \times 10^{-4} \times D^{-1}$	$0.0128 \times D^{-\frac{1}{2}}$	$0.0107 \times D^{\frac{1}{2}}$	-	$0.02388 \times D^{\frac{3}{2}}$				$12087 \times D^{-1}$	-
dependence on resolution d	-	-	-	$\alpha\ 1/d^2$	-	-	-	$\alpha\ 1/d$	$\alpha\ 1/d$	$\alpha\ 1/d$				-	$38{,}000/d$

Parameters in electron microscopy of single protein molecules or molecular assemblies. To simplify the presentation, it is assumed that the molecules are arranged in a closely-packed 2-dimensional crystal with a square unit cell as shown in Fig. 3. The formulae used to derive Table 2 are given in the Appendix.

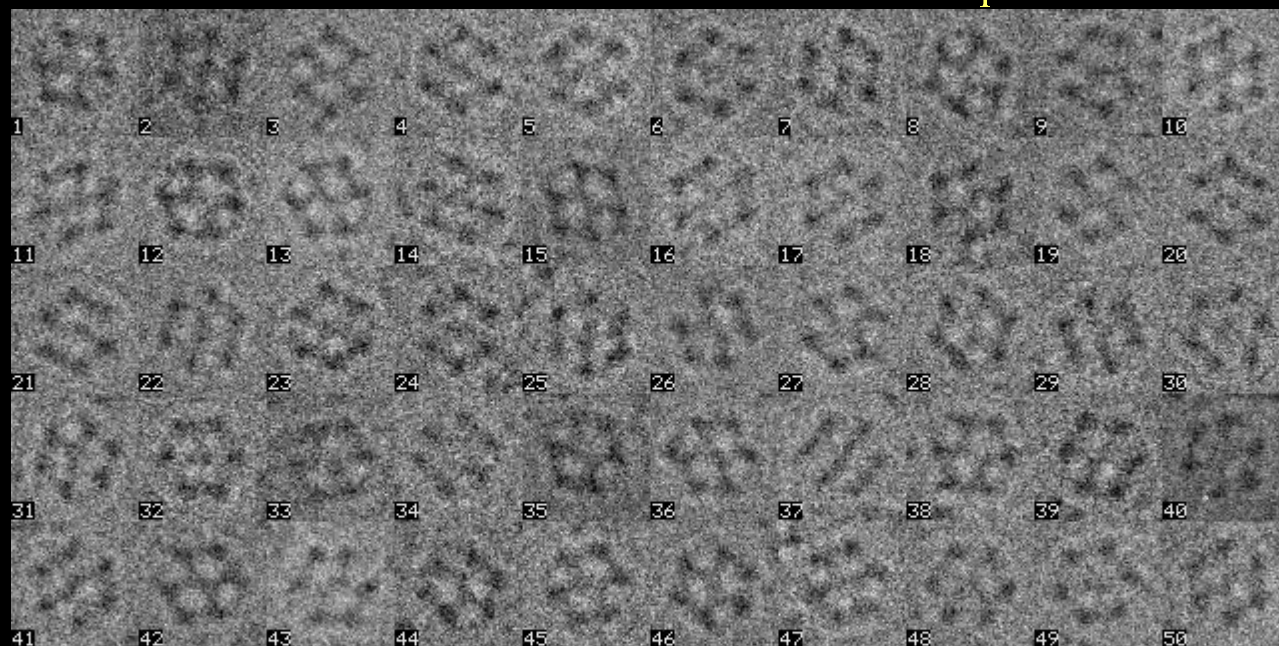
Number of particles needed to reach given resolution as a function of B-factor

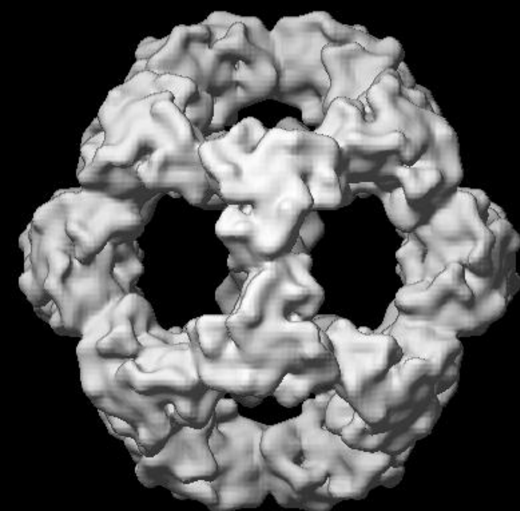


Rosenthal tilt pair validation test

UNTILTED

$(\psi,\theta,\varphi)_u$

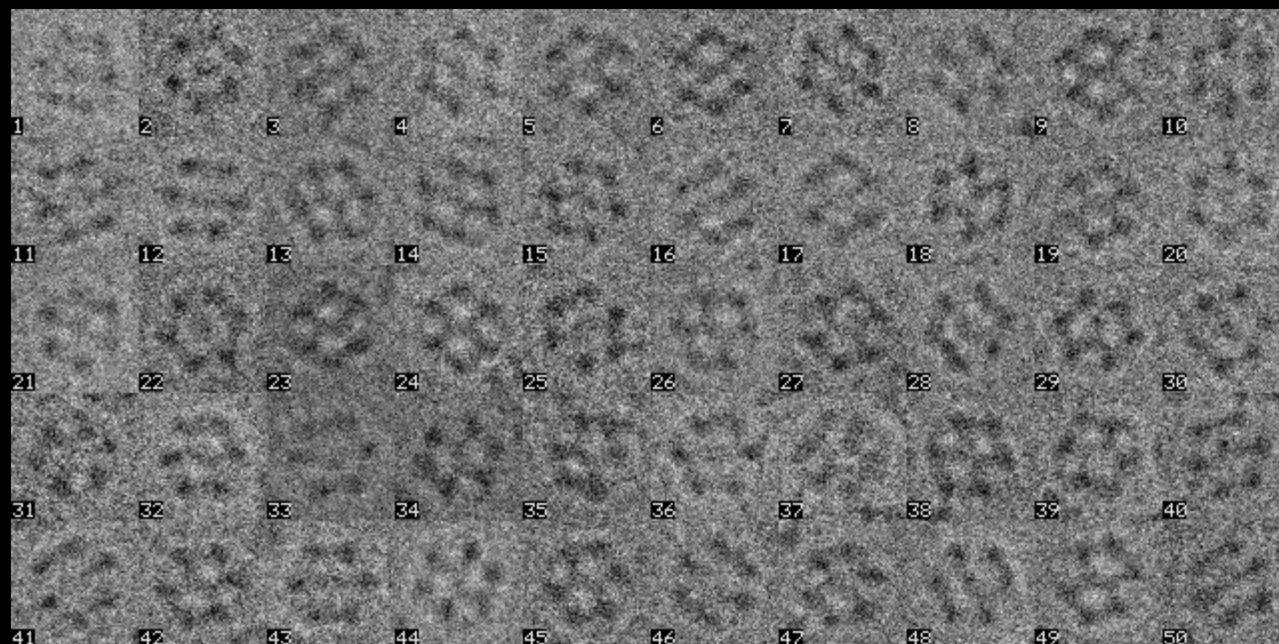


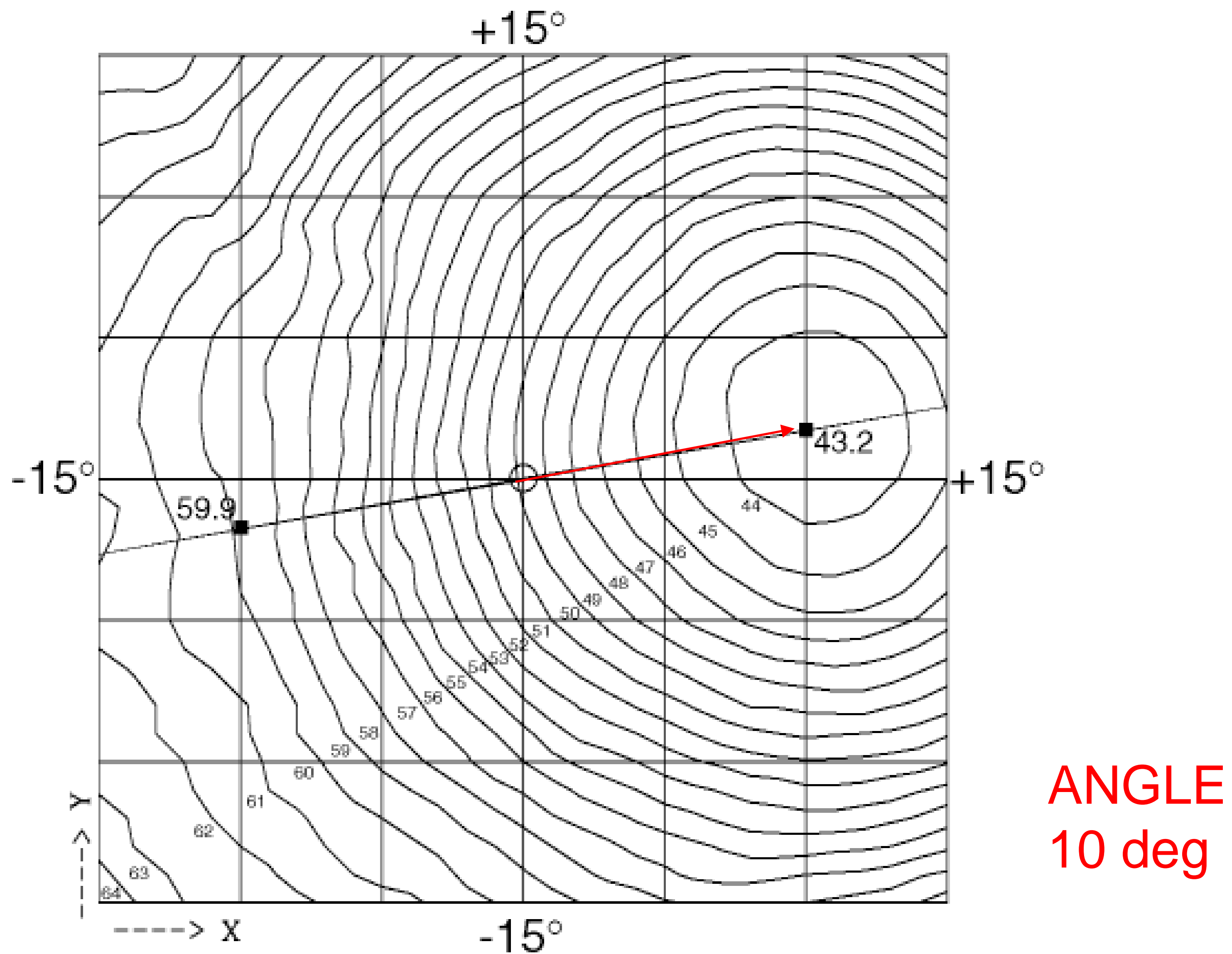


TILTED

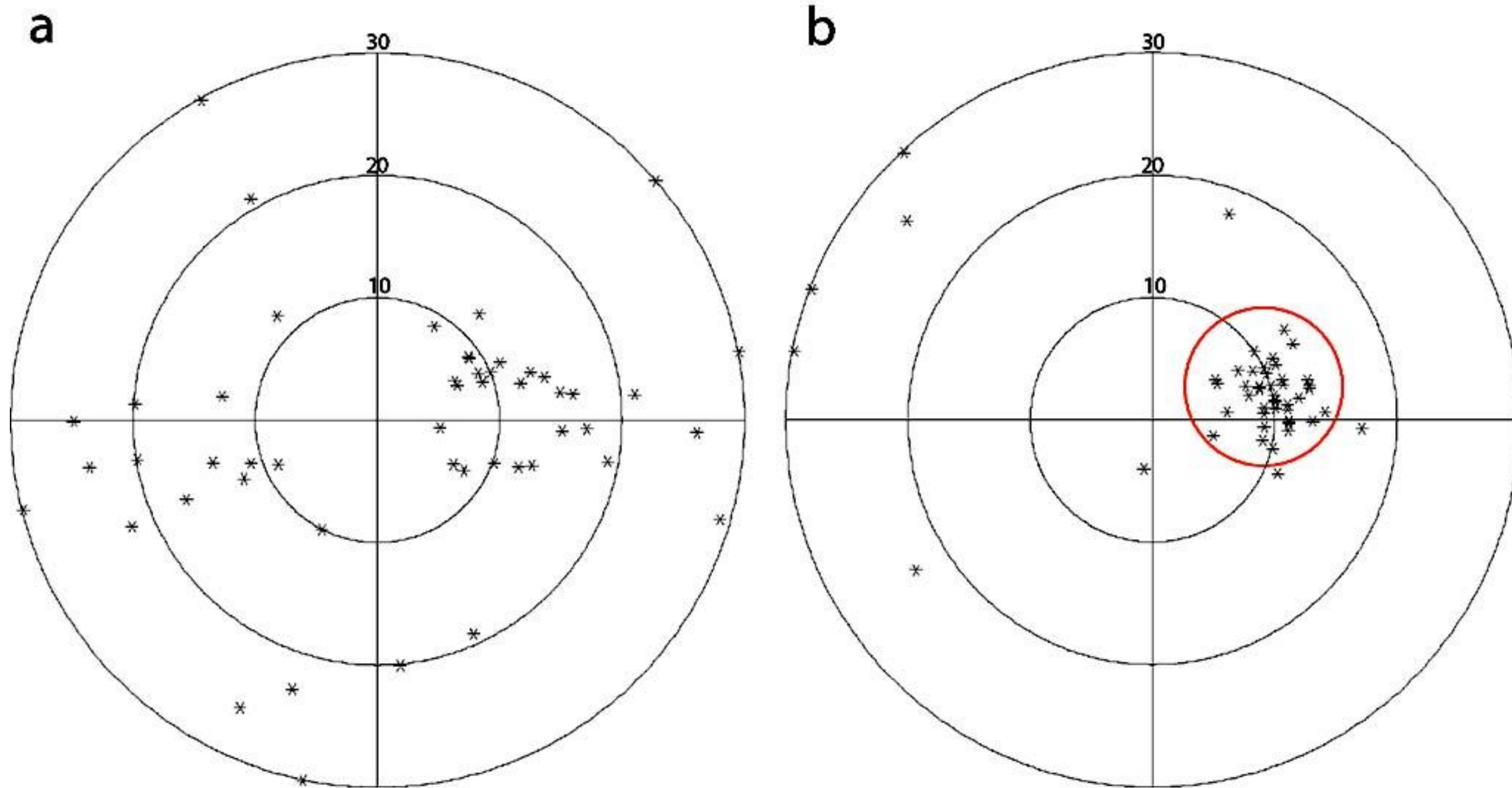
10 degrees

$(\psi,\theta,\varphi)_t$





Mean phase residual for 50 particle image pairs – ANGPLOT + FREALIGN



Individual particle image pairs – TILTDIFF output

Application of Rosenthal & Henderson tilt pair validation approach

(9/90 citations)

- Pyruvate dehydrogenase : R & H (2003) JMB **333**, 721-42
- *Neurospora* P-type ATPase : Rhee et al (2002) EMBO J. **21**, 3582-89
- Bovine ATPase : Rubinstein et al (2003) EMBO J. **22**, 6182-92
- Chicken anaemia virus : Crowther et al (2003) J.Virol. **77**, 13036-41
- HepB surface antigen : Gilbert et al (2005) PNAS **102**, 14783-88
- Hsp104, yeast AAA+ ATPase : Wendler et al (2007) Cell **31**, 1366-77
- Yeast ATPase : Lau et al (2008) JMB **382**, 1256-64
- V-type ATPase, *T.thermophilus* : Lau/Rubinstein (2009)
- DNA-depend PKase : Williams et al (2008) Structure **16**, 468-77

Conclusion

Contributions of different factors to contrast loss

- Radiation damage degrades structure factors — $\Delta B = 80$
- Detectors (e.g. film) poor high resolution MTF (and DQE) — $\Delta B = 60$
- Charging and mechanical movement — $\Delta B = 60$ to 500

- Intrinsic molecular flexibility — $\Delta B = 30$ to 500

Technical challenge is to reduce contribution of everything except radiation damage to near zero

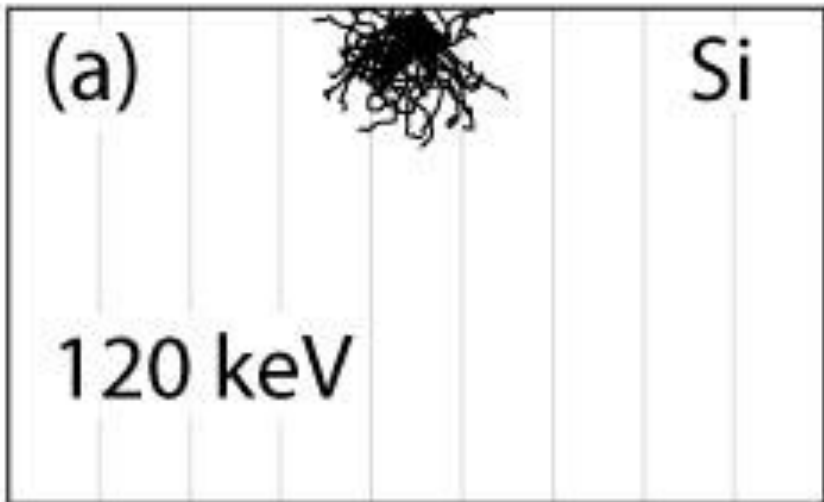
Detectors at present

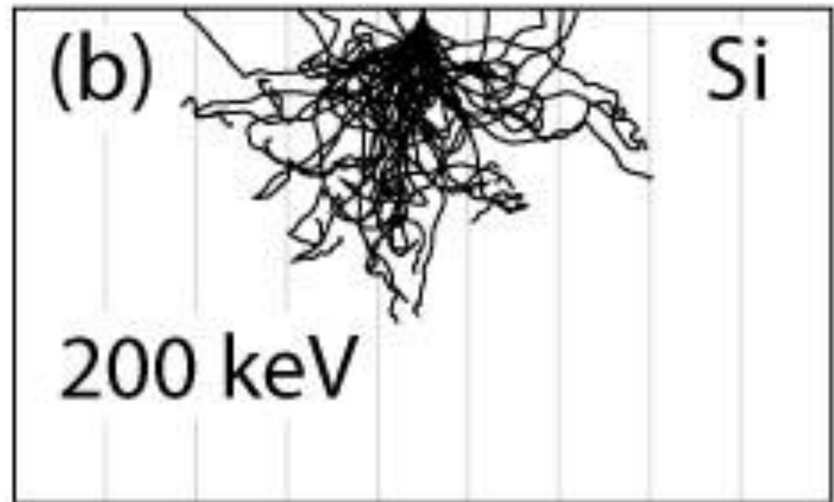
- Film (SO-163)
- Phosphor/Fibre Optics/cooled CCD
- Phosphor/Lens/cooled CCD

Prototype detectors

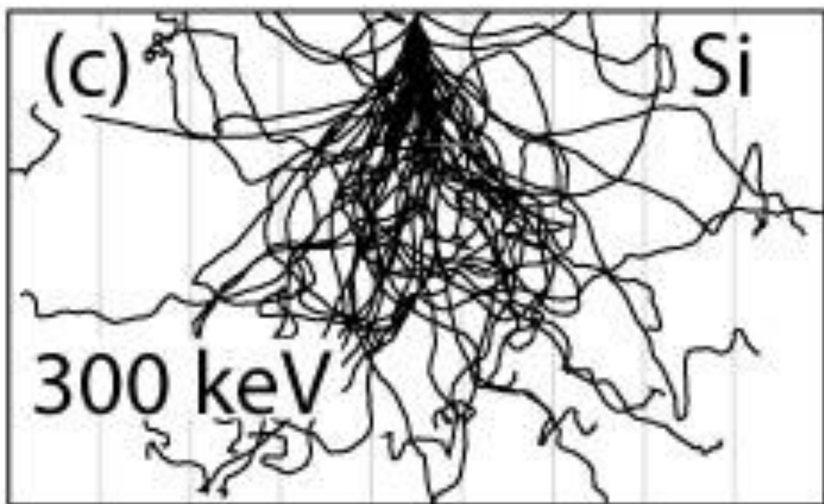
- Hybrid Pixel Detectors (Medipix)
- Monolithic Active Pixel Sensors (MAPS/CMOS)

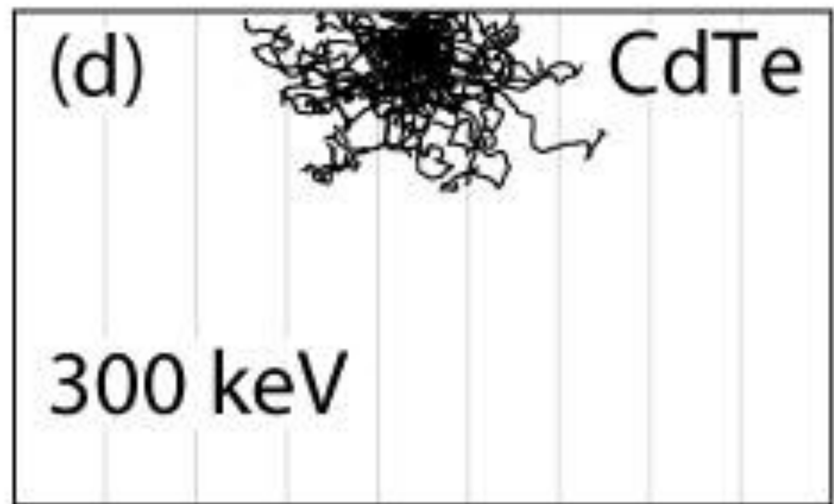
Electron tracks - Monte Carlo simulation



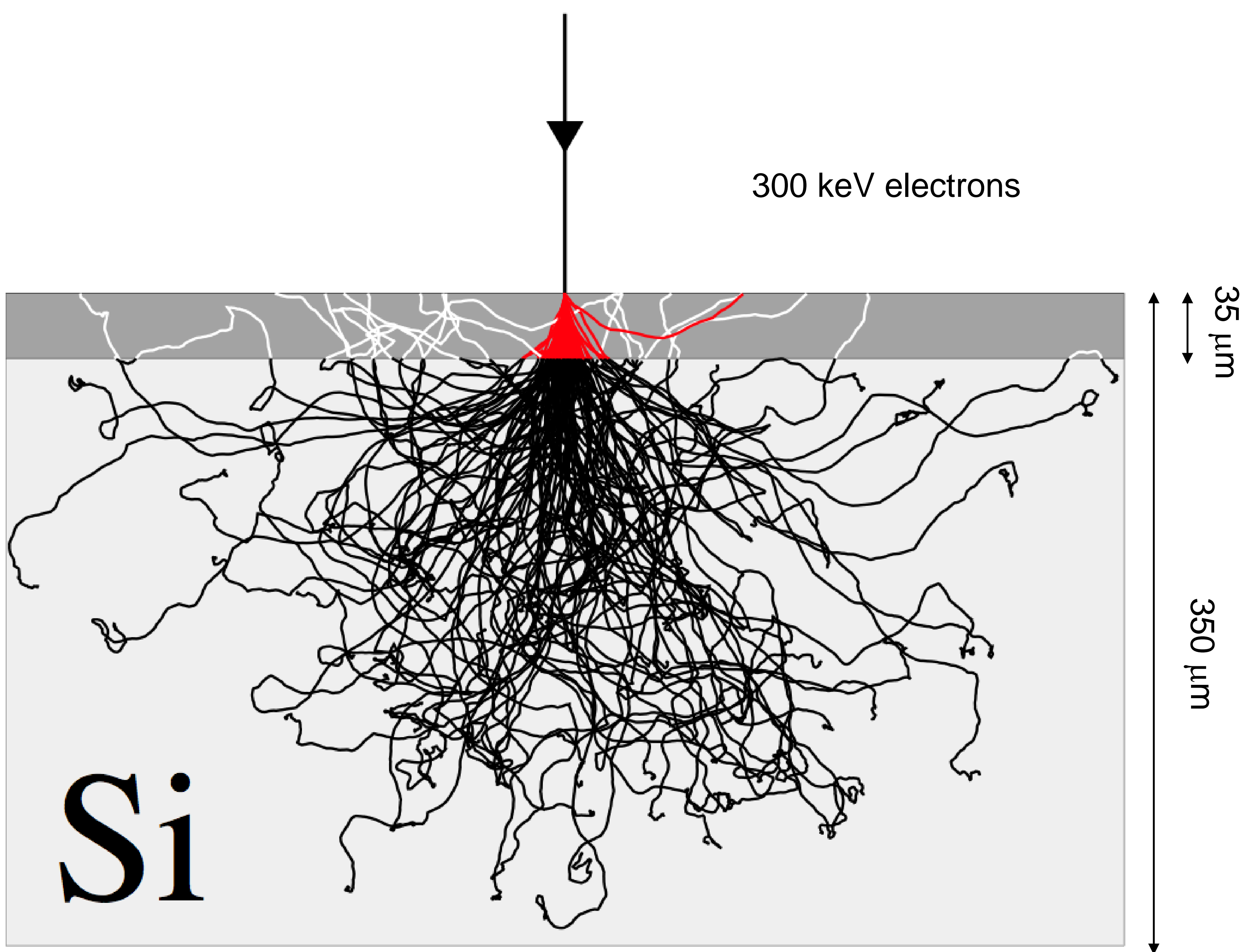


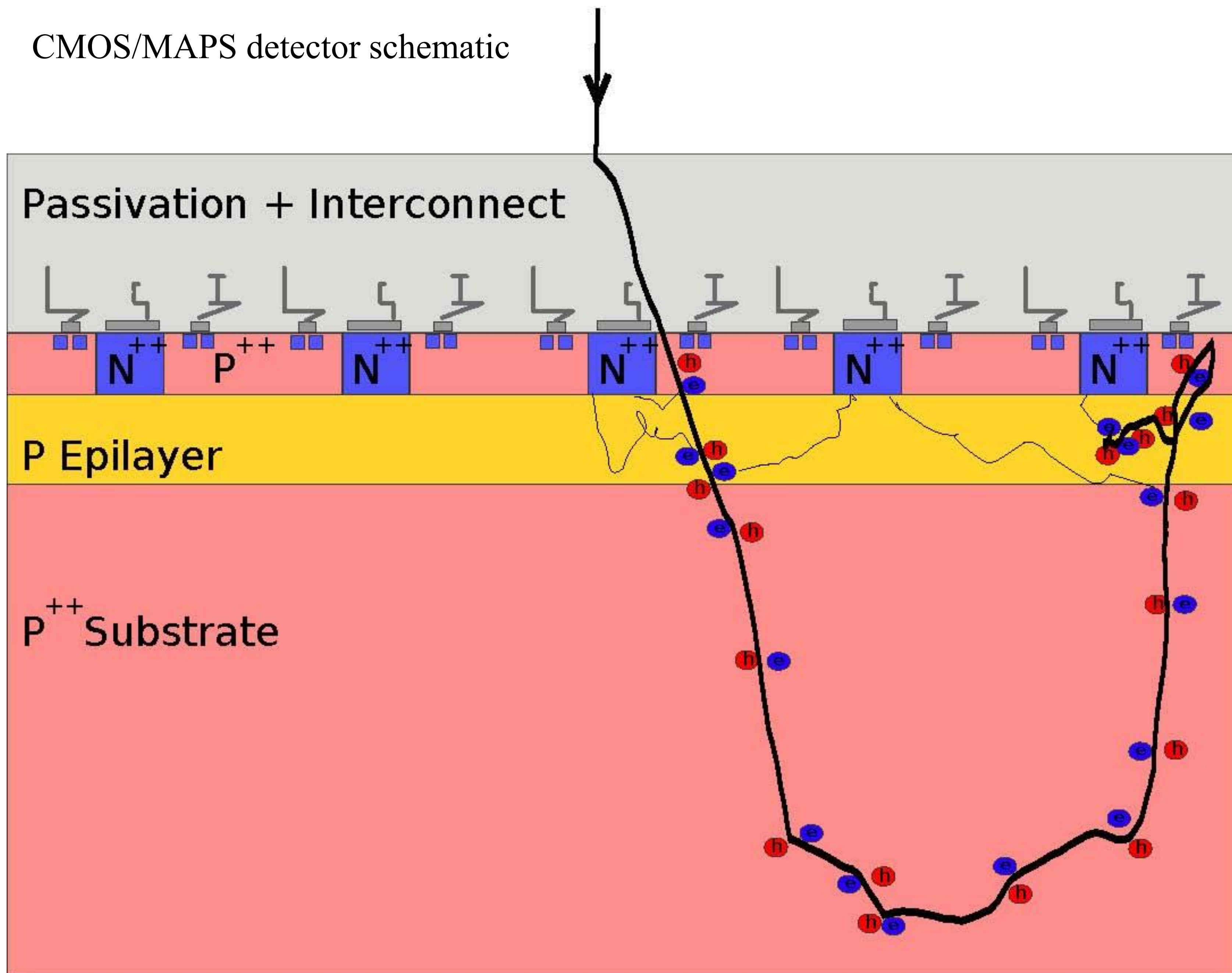
300 μm

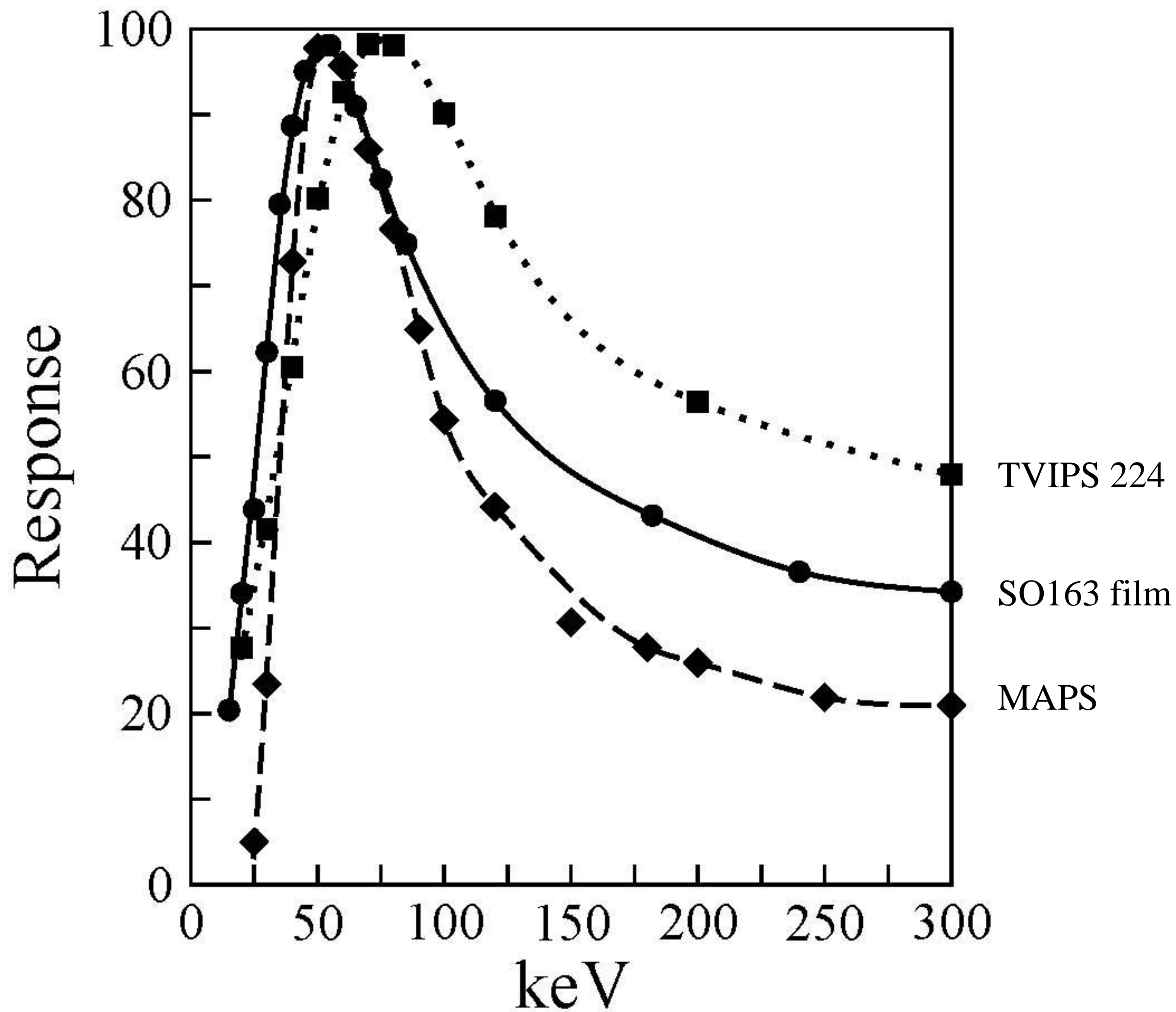




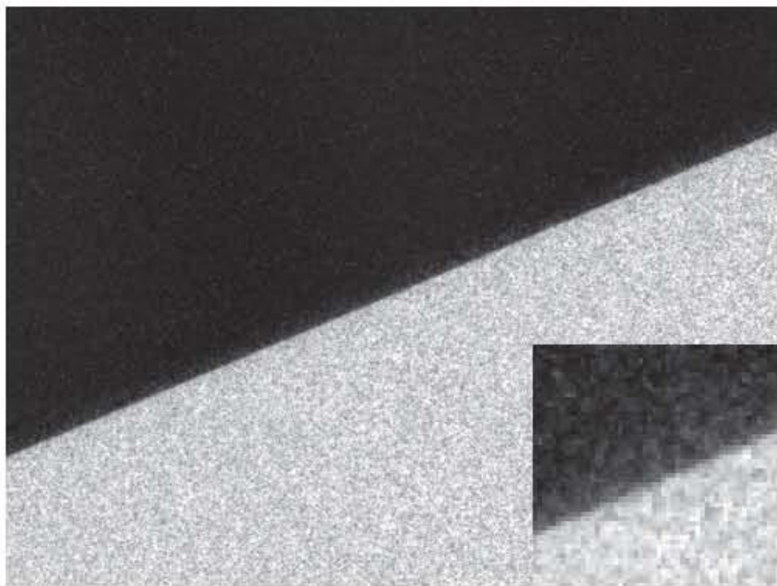
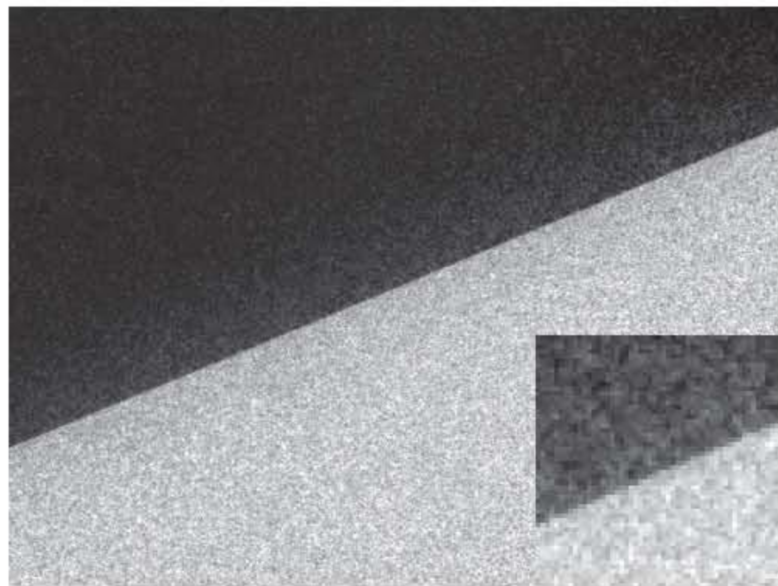
55 μm


300 keV electrons
35 µm
350 µm
Si

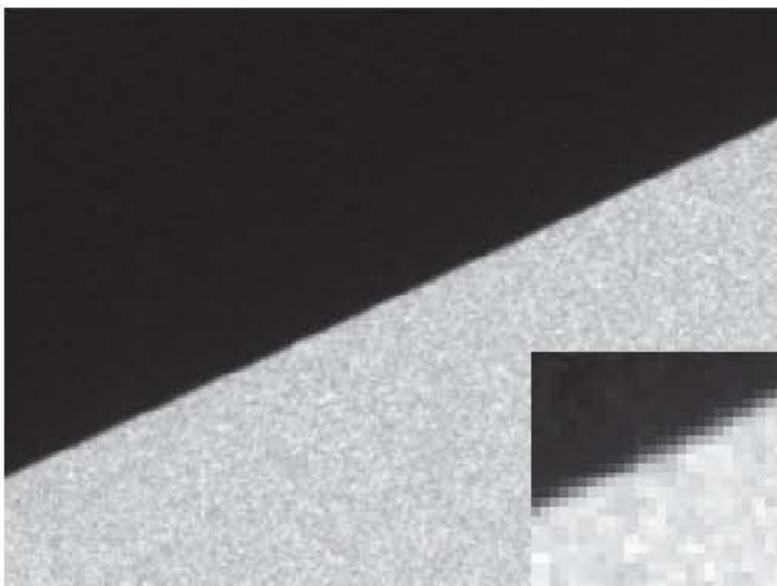
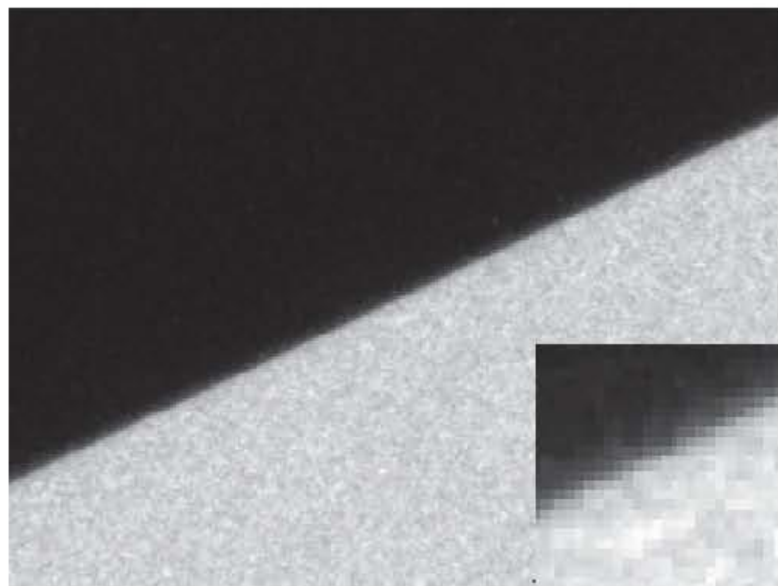

CMOS/MAPS detector schematic
Passivation + Interconnect
N++
P++
P Epilayer
P++ Substrate



Response
keV
TVIPS 224
SO163 film
MAPS
0
20
40
60
80
100
0
50
100
150
200
250
300

(a) 120kV SO-163 film 300kV (b)

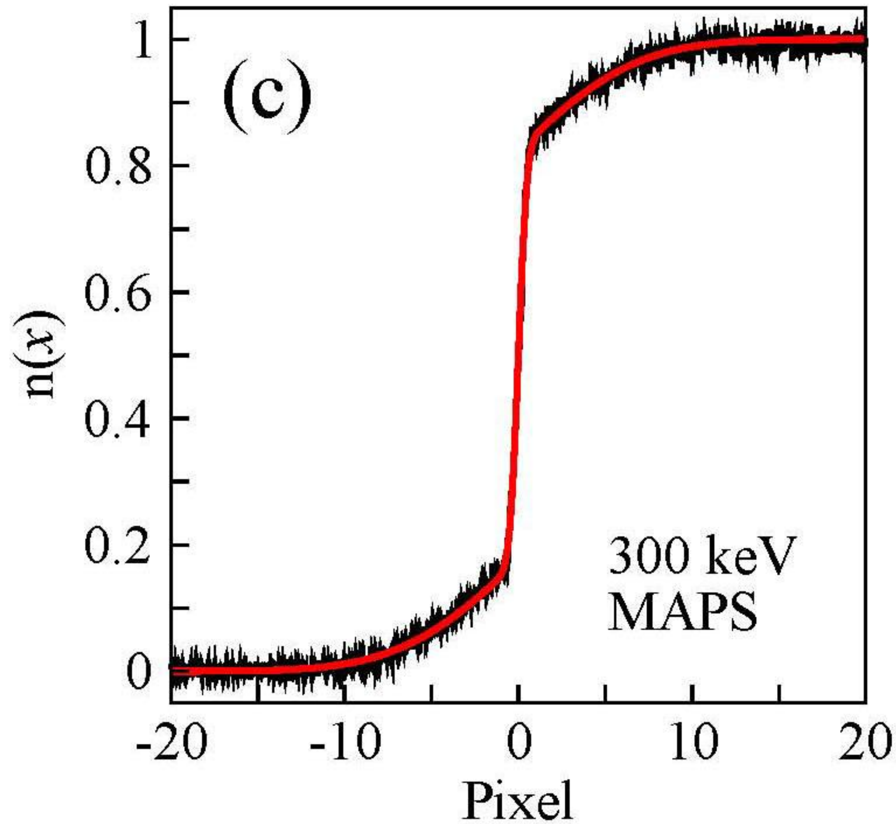



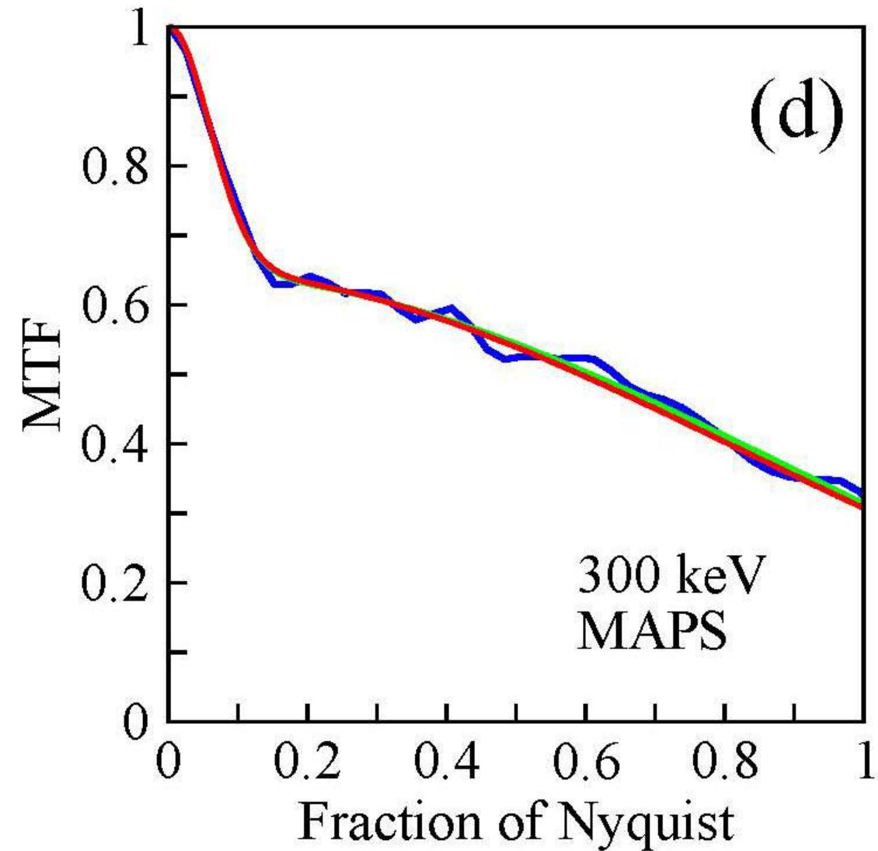
(c) TVIPS 224 (d)

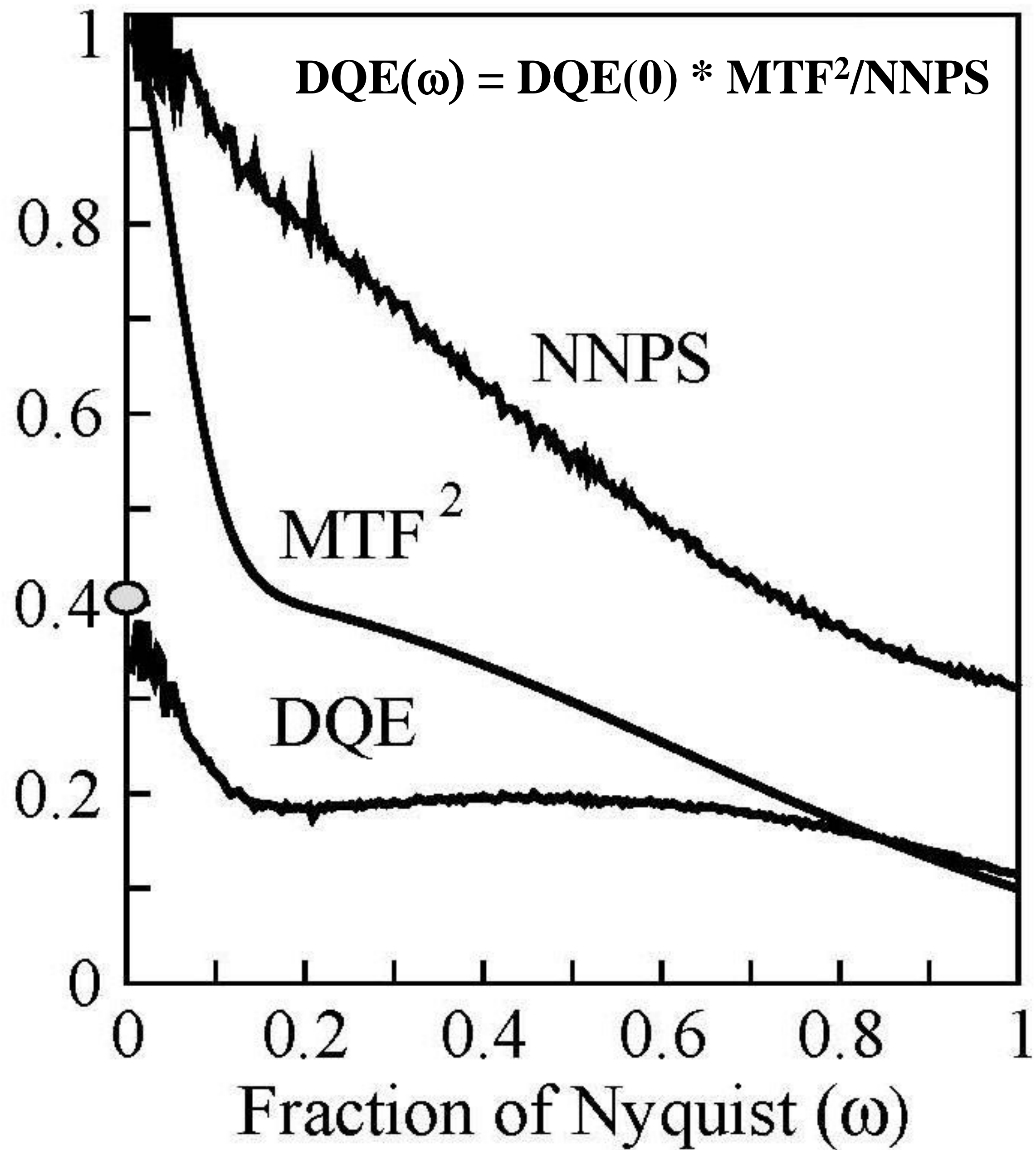
MTF

Double Gaussian fit to raw data

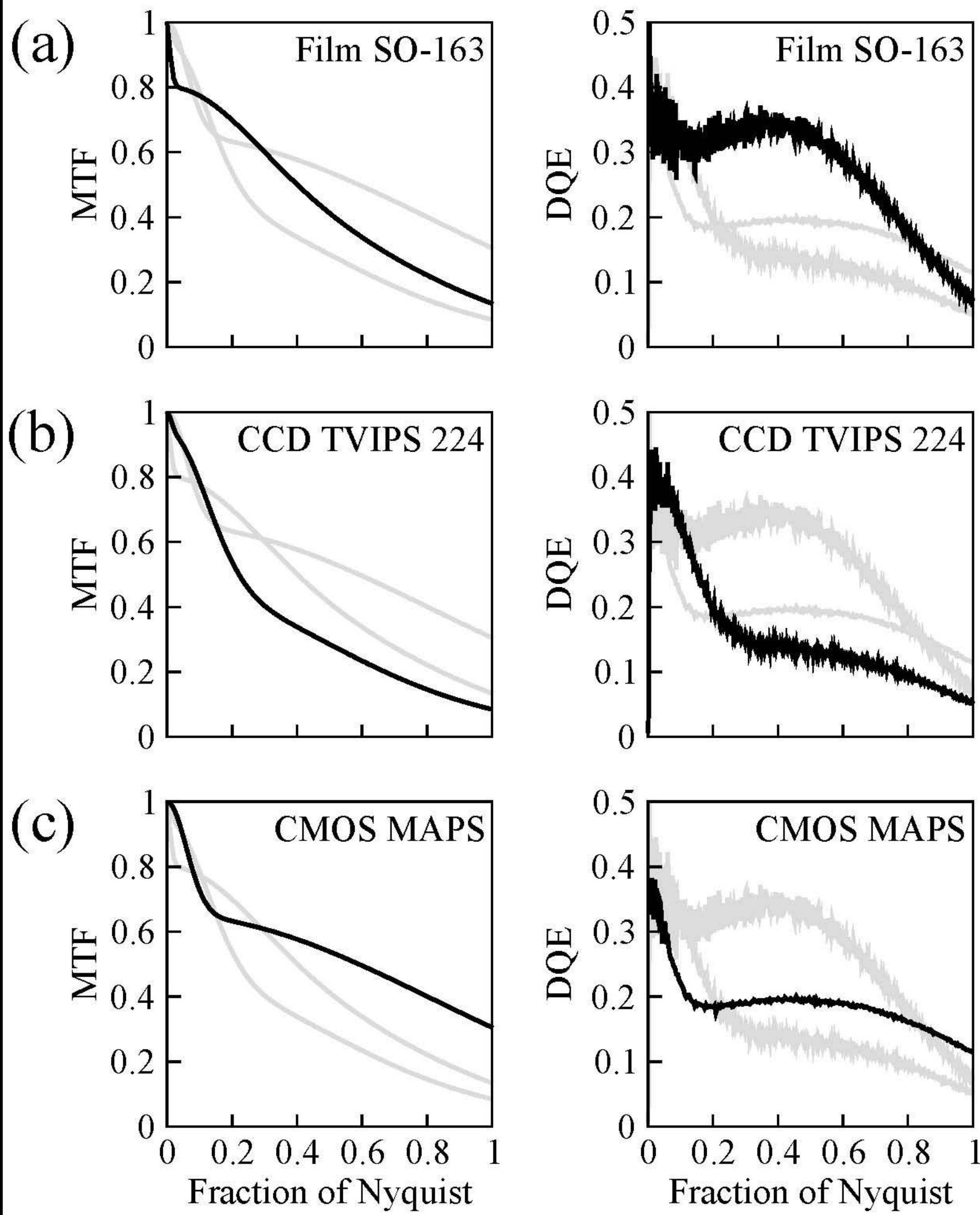
MTF from fit and by differentiation

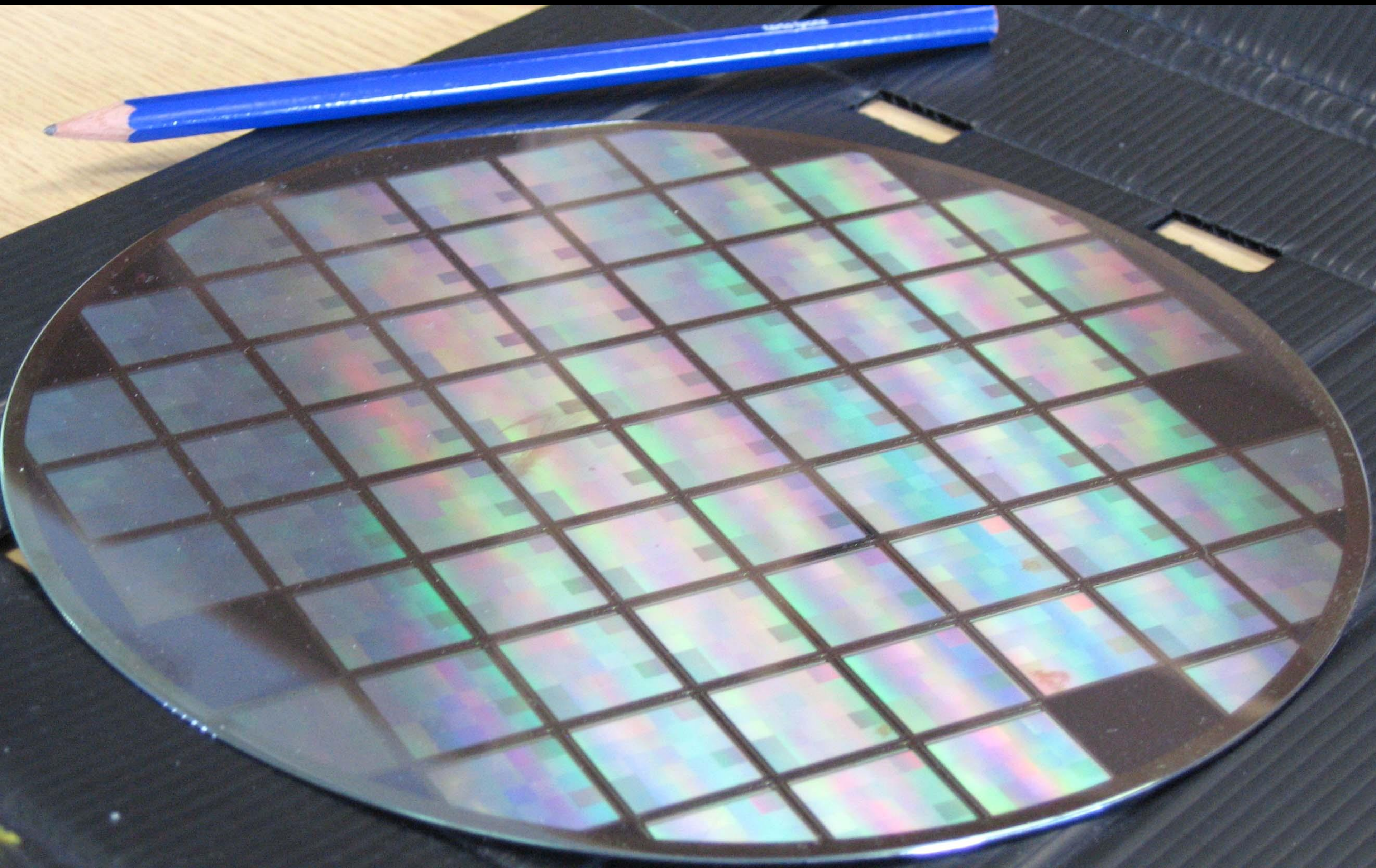


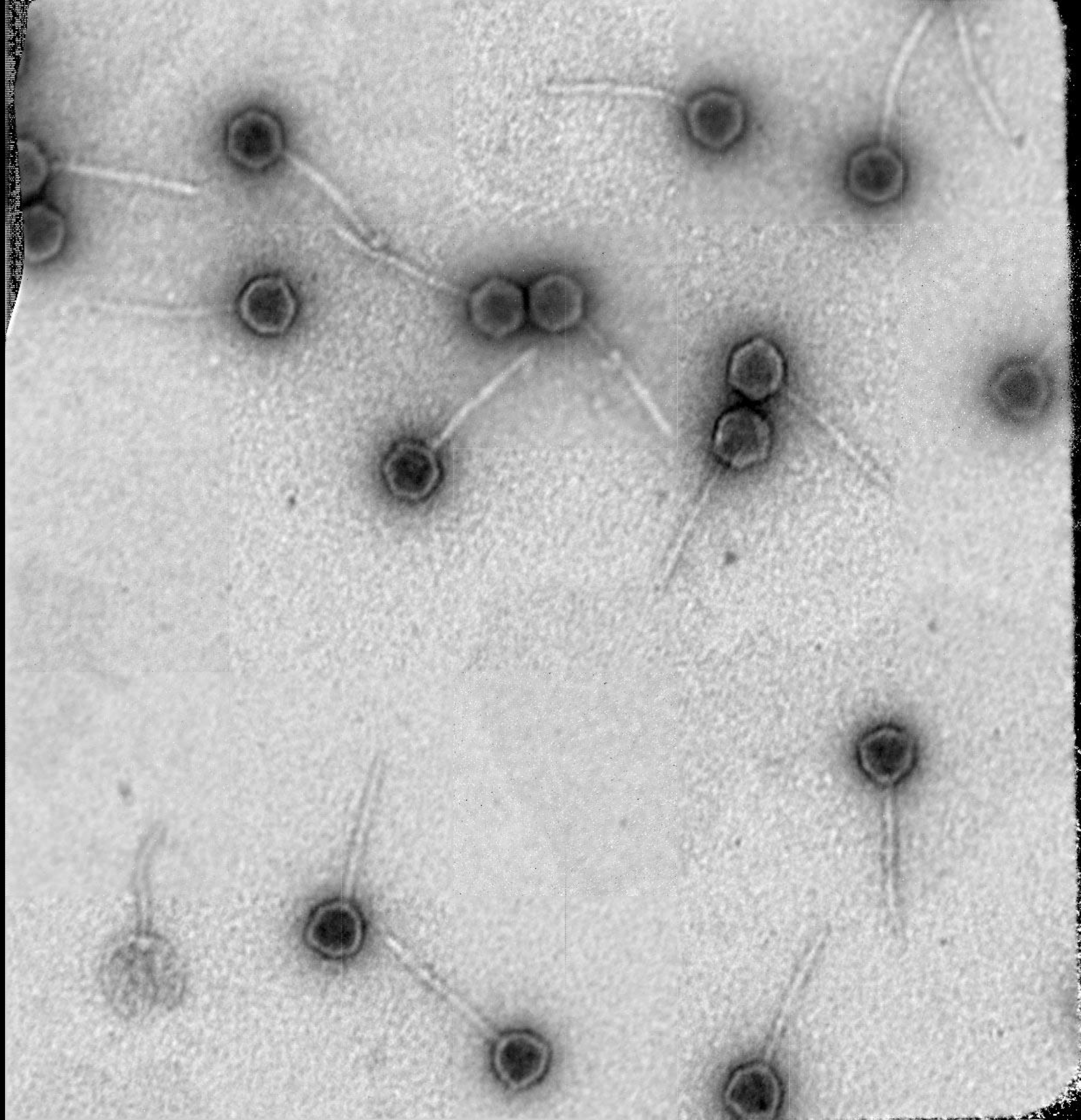




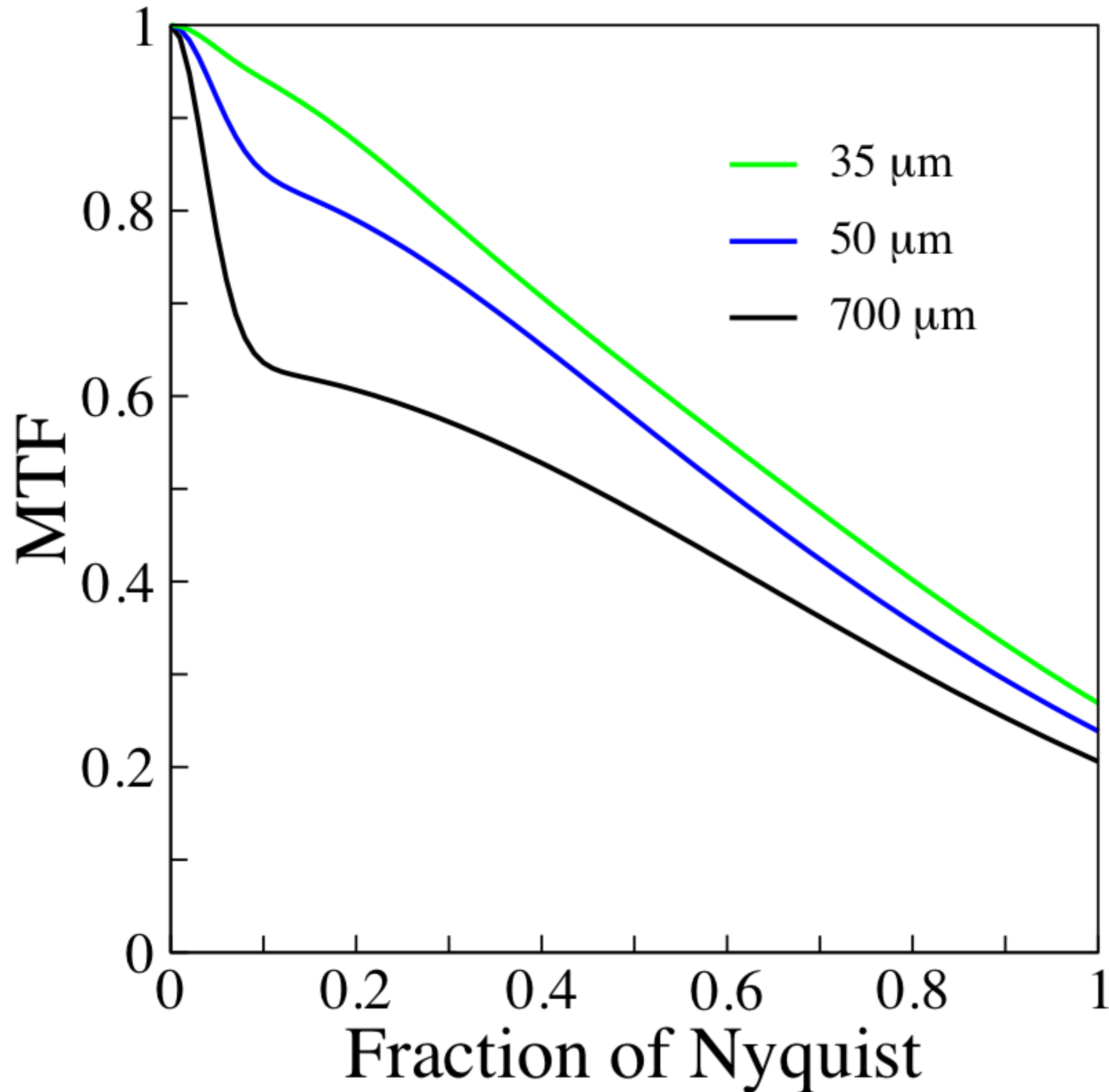
MAPS
300kV

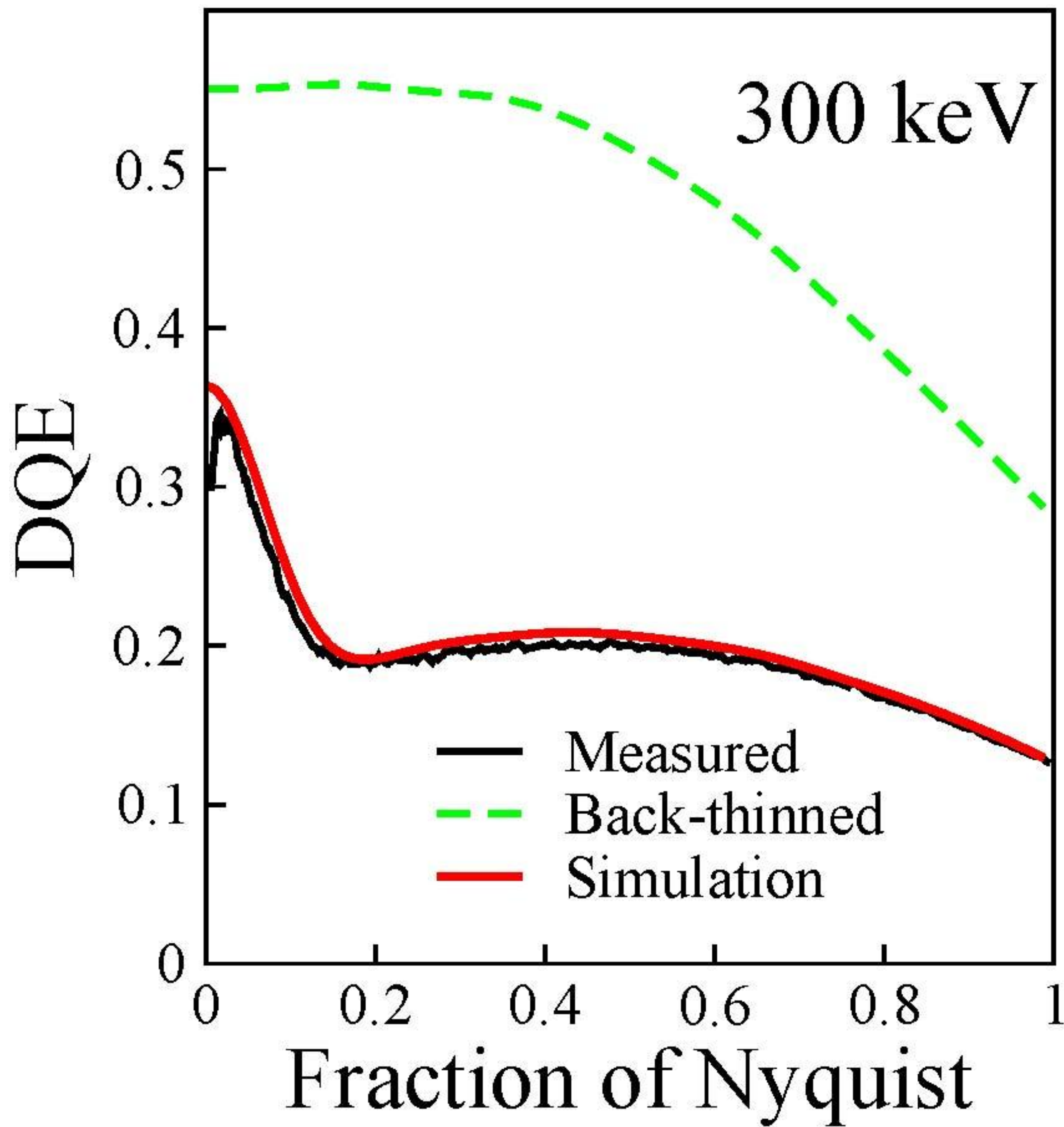

300 keV
(a)
Film SO-163
MTF
DQE
(b)
CCD TVIPS 224
(c)
CMOS MAPS
Fraction of Nyquist
0
0.2
0.4
0.6
0.8
1
0.1
0.3
0.5



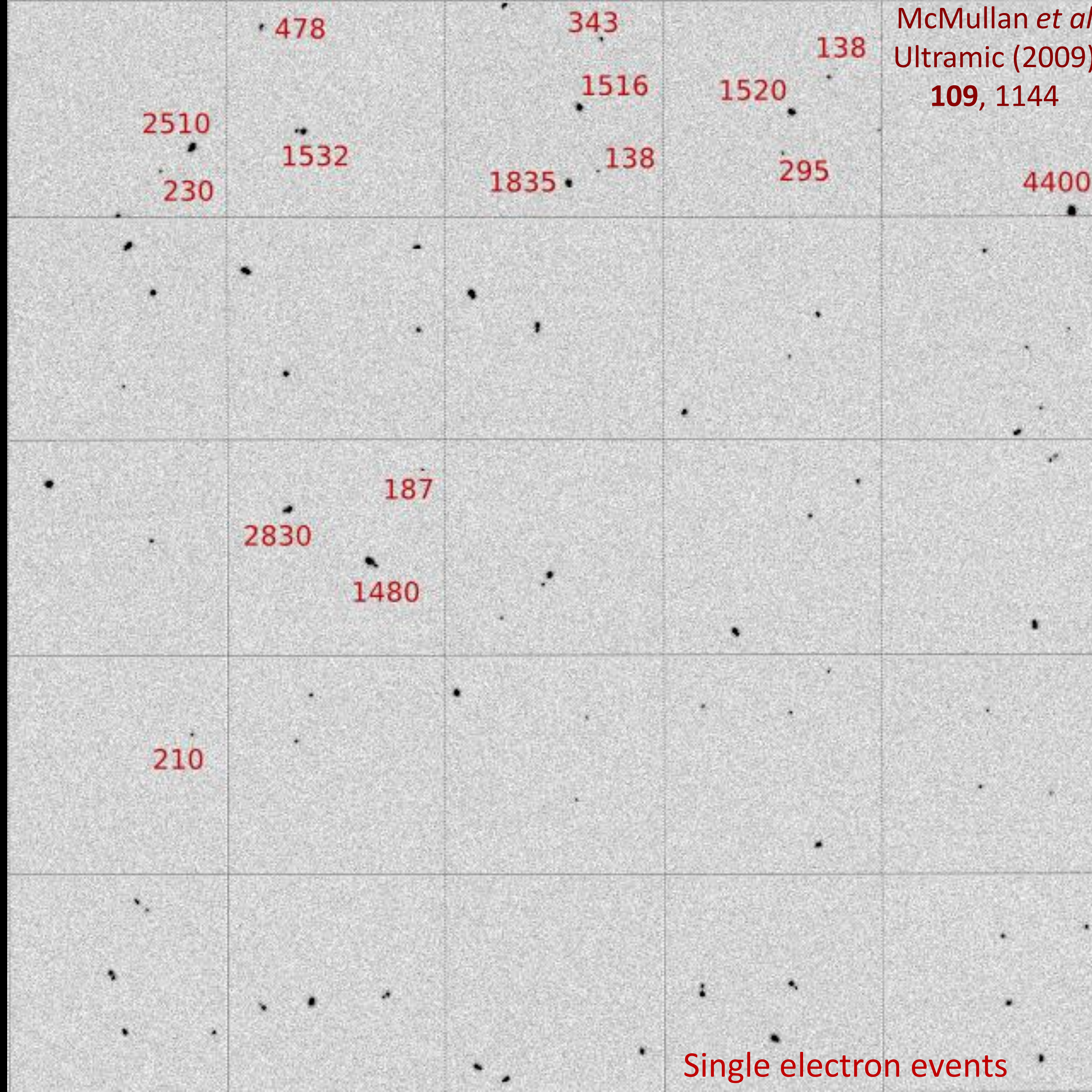


Effect of backthinning





MAPS backthinning simulation


McMullan et al
Ultramic (2009)
109, 1144
478
343
138
1516
1520
2510
1532
138
295
230
1835
4400
187
2830
1480
210
Single electron events

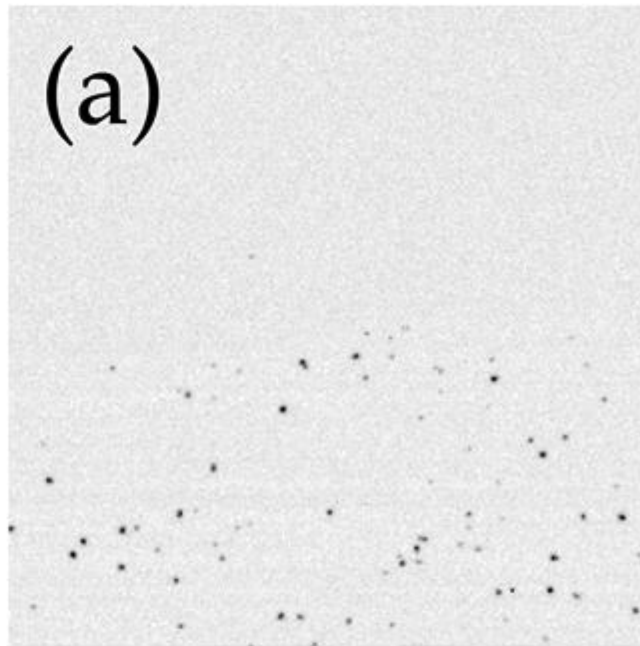
Electron counting

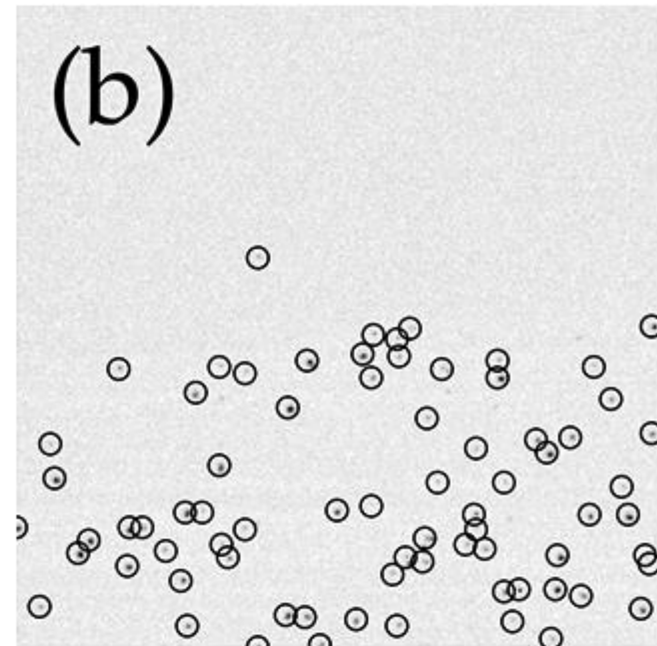
(a) Raw frame

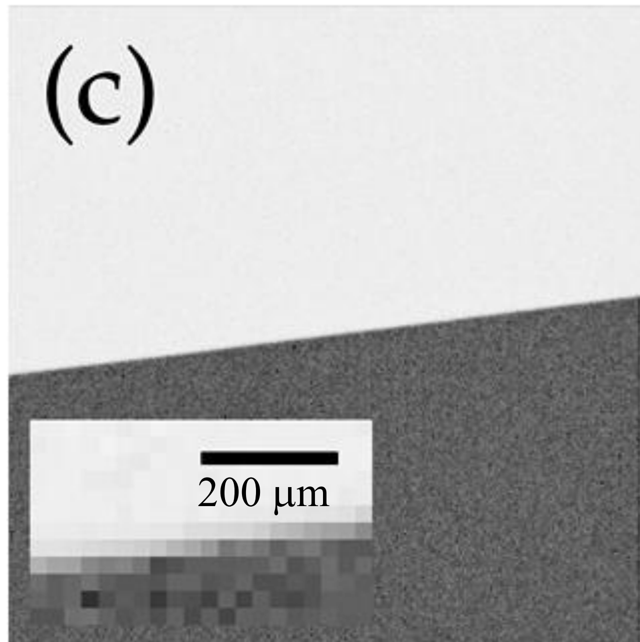
(b) Identified events

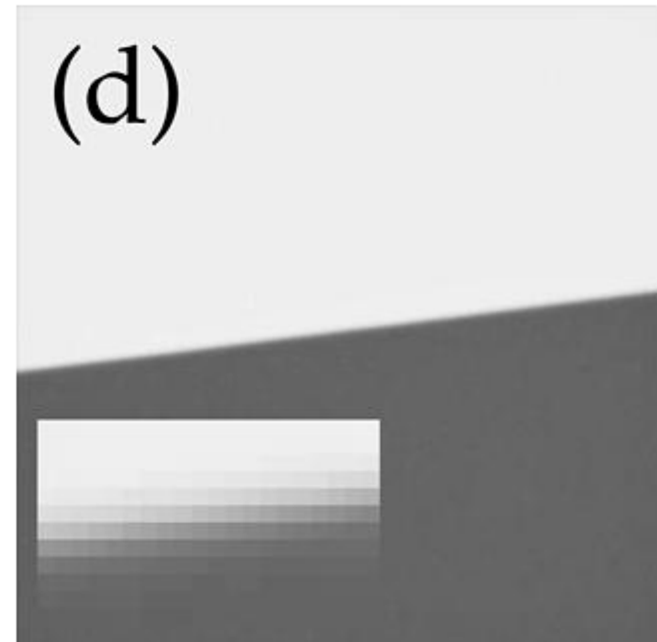
(c) Counting mode (70,000 frames)

(d) Integrating mode (same dose)



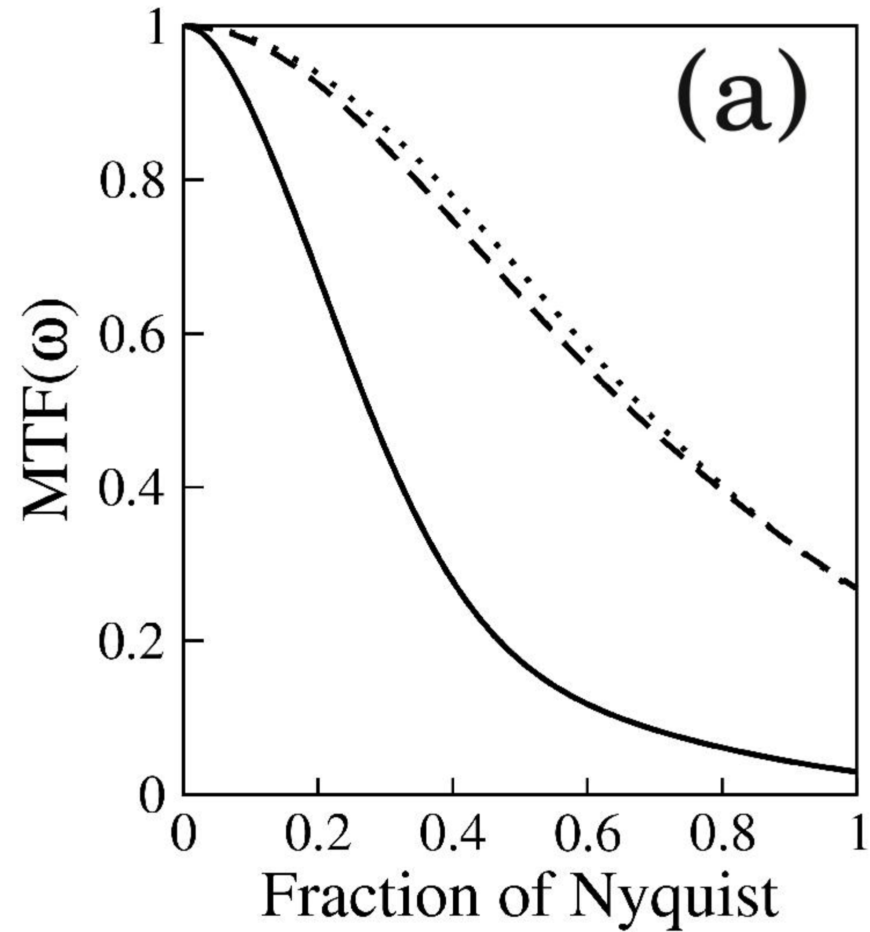


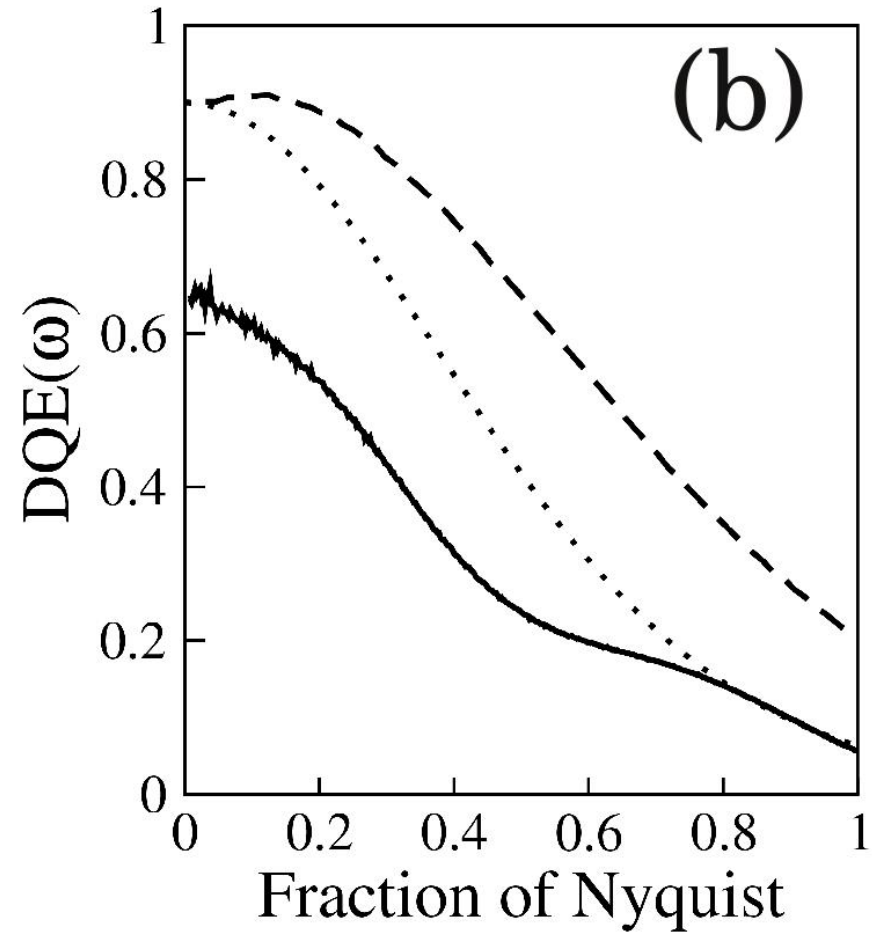




McMullan et al, Ultramic (2009) **109**, 1411

Integrating mode
Renormalising mode
Peak pixel mode





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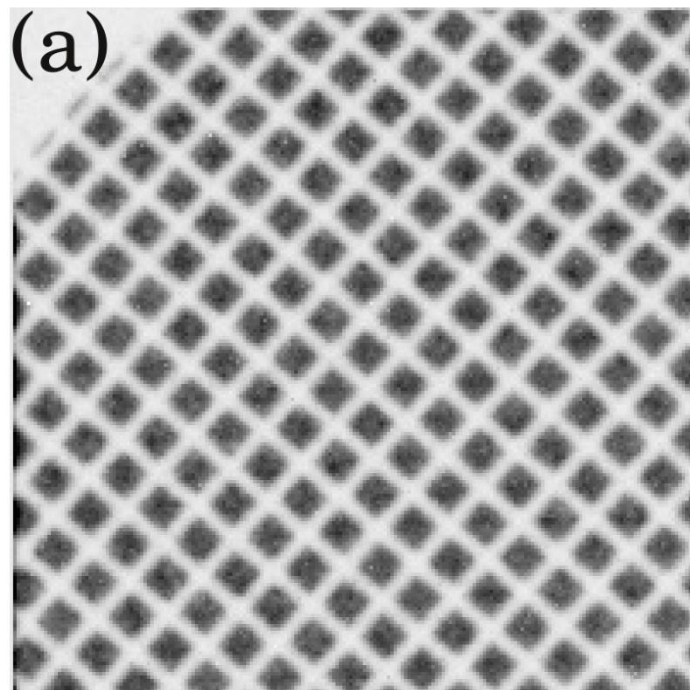
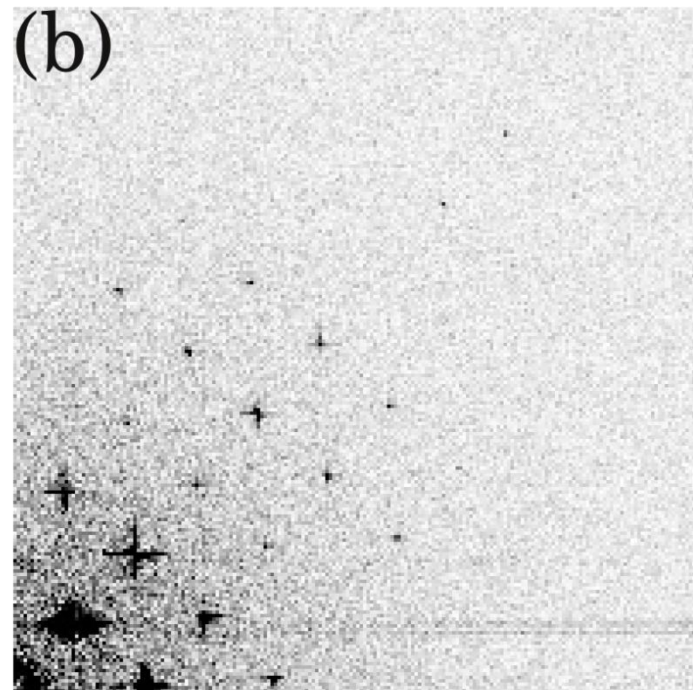
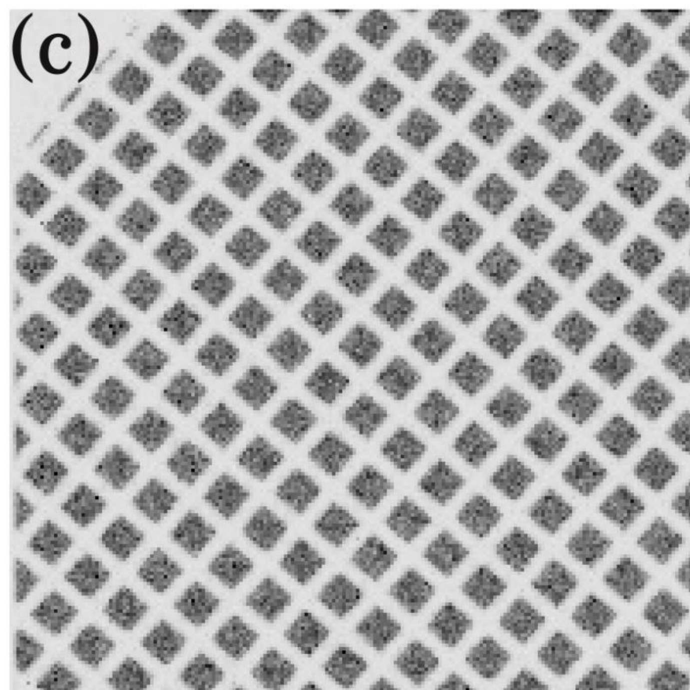
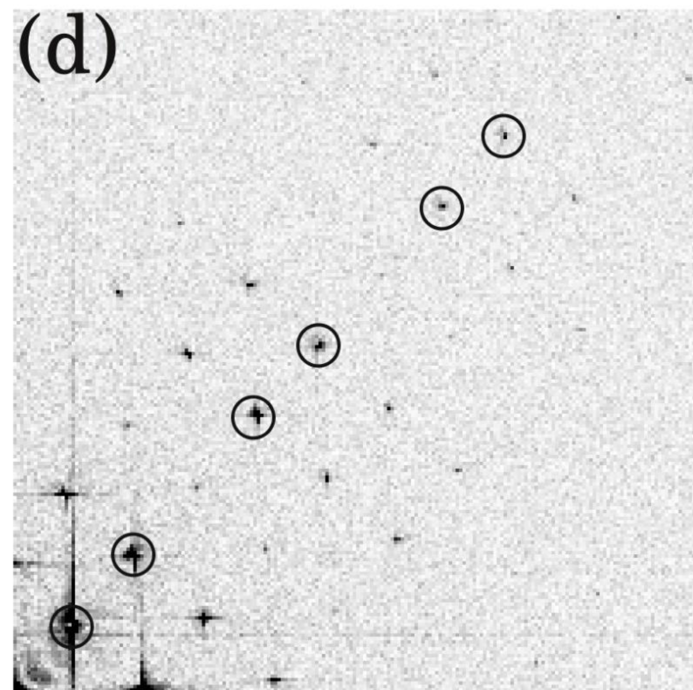
Integrating Mode
5 frames
in 0.1 sec





Single electron mode
7500 frames
in 50 sec





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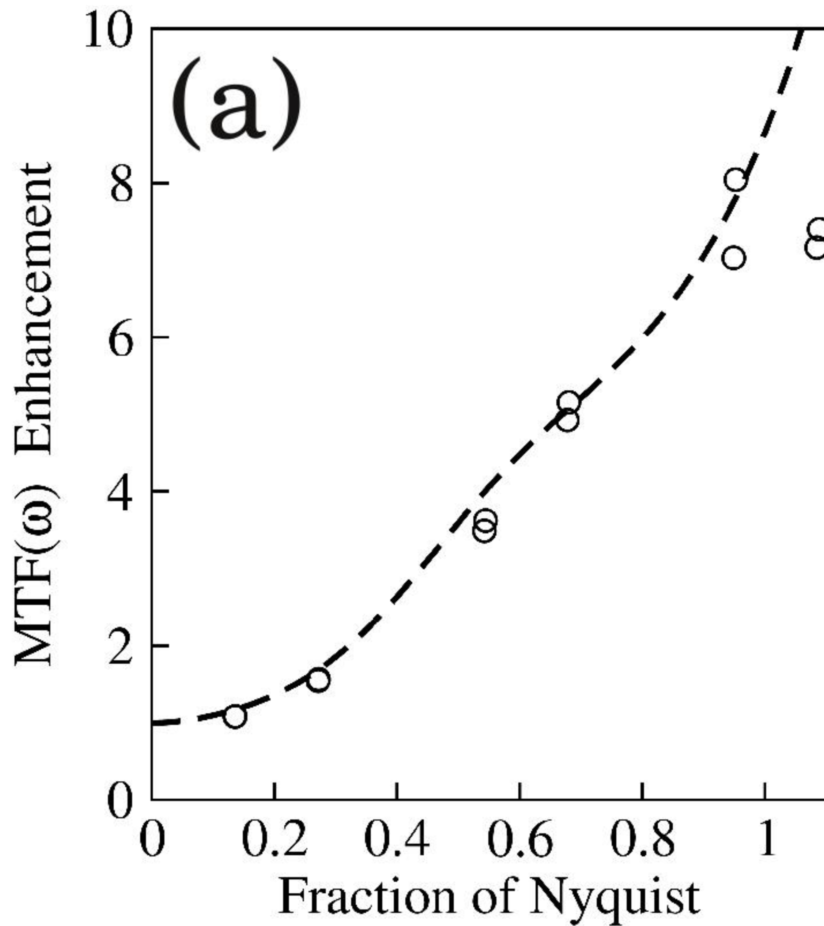
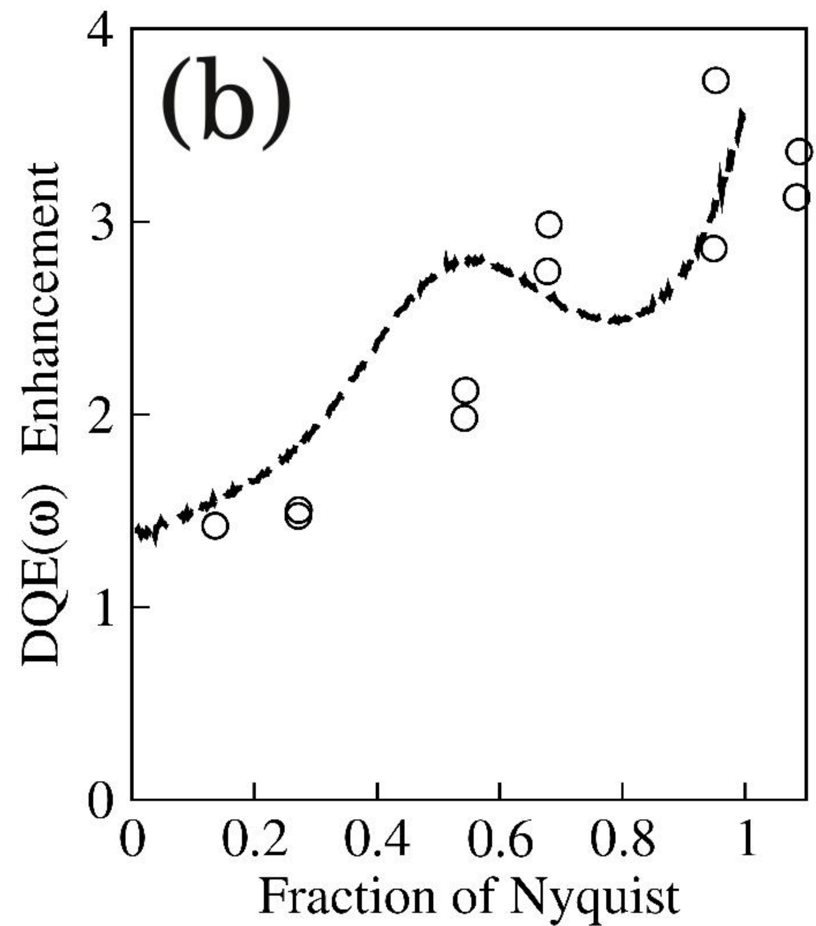
Enhancement of MTF and DQE by renormalisation of individual electron events circles from grid image, lines from edge image





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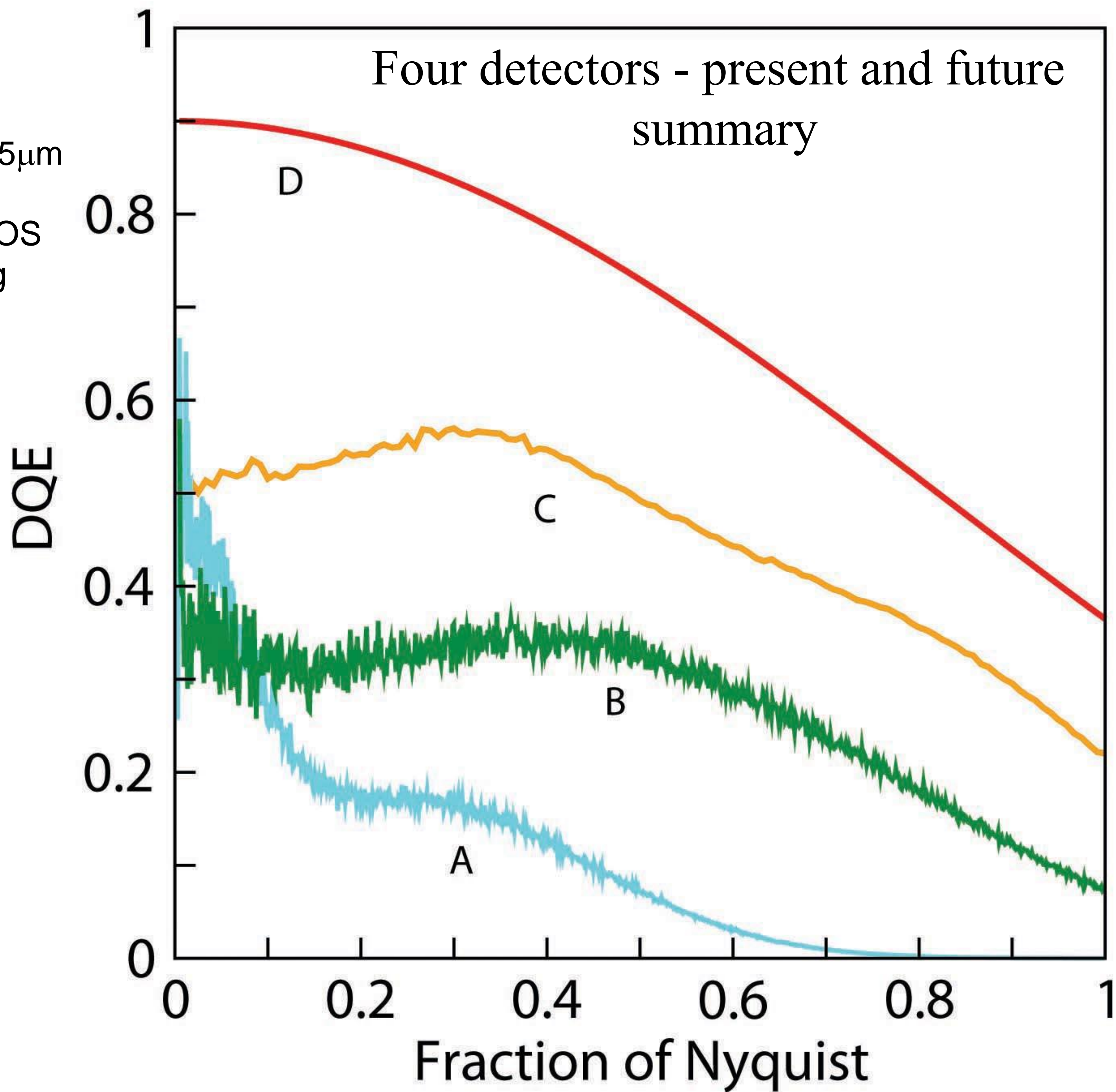
A Ultrascan 4000 15µm
B SO-163 film 7µm
C Backthinned CMOS
D Electron counting



Bridget/Clint/Ron's 12 Questions -- A

- Will we get to atomic resolution with particles other than viruses? Yes
- Is an atomic resolution 3D map by single particle analysis worth the effort? Yes
- Can single particle work compete with other approaches? Yes
- What resolution is useful? 40, 20, 8, 4 Ångstroms

Questions -- B

- What can we NOT do by the single particle approach?
  Not small, not unstructured, not flexible with small domains

- Are there possibilities for improving the result by better freezing? Maybe but not yet clear how

- Are there new ways to reduce radiation damage?
  Good stable environment, deuteration, but effects are minor

- How do we identify bad images? Only one type of good image
  Hundreds of kinds of bad image

Questions -- C

- What specimen preparation methods can we design to minimise heterogeneity before we get to the microscope?
  Investigate adding ligands, making complexes, selecting mutations to create homogeneous population
- Can we get clean well-characterized specimens?
  Good standard biochemistry, e.g. protein purified for X-ray xtlog tend to give very clean cryoEM grids
- Can we stabilise a complex with ligands or other additives? Yes
- Should we use glutaraldehyde or other bifunctional cross-linking reagents to prevent subunit loss or to stabilise conformations? Understand why Grafix works so well
  – must be stresses either during blotting or during freezing

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