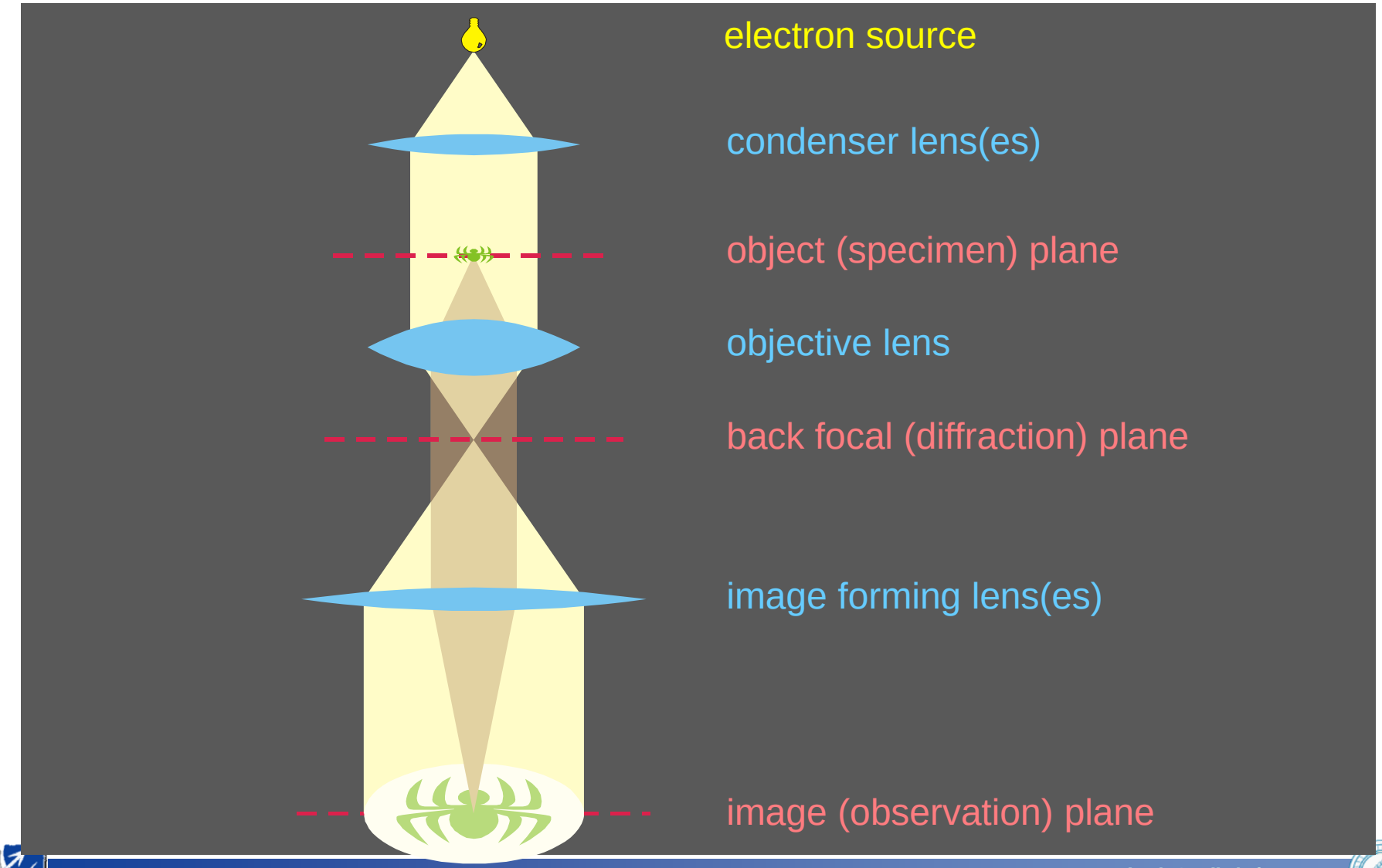
Phase plates for cryo-EM

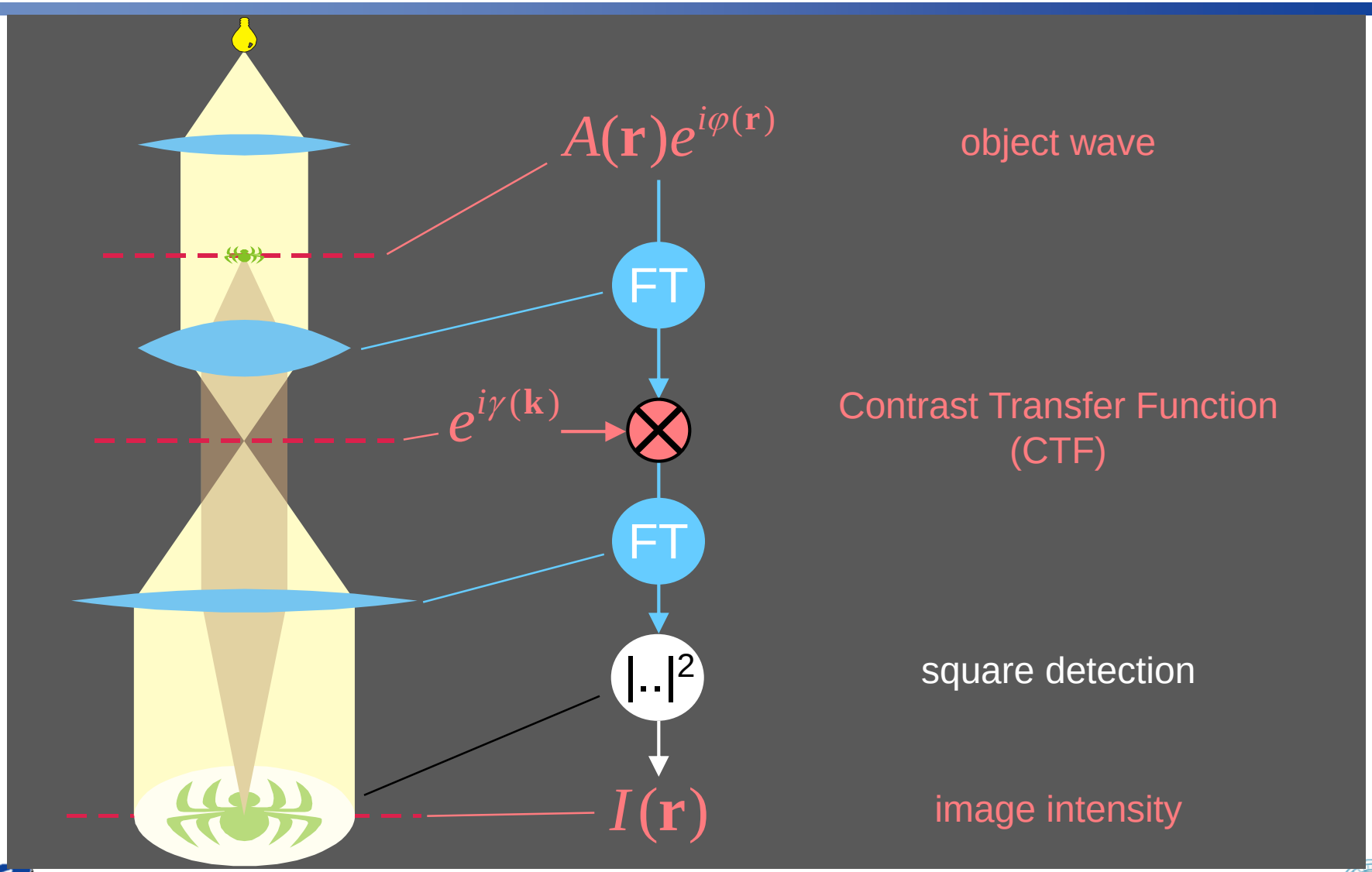
Rado Danev

Max Planck Institute of Biochemistry, Martinsried, Germany.

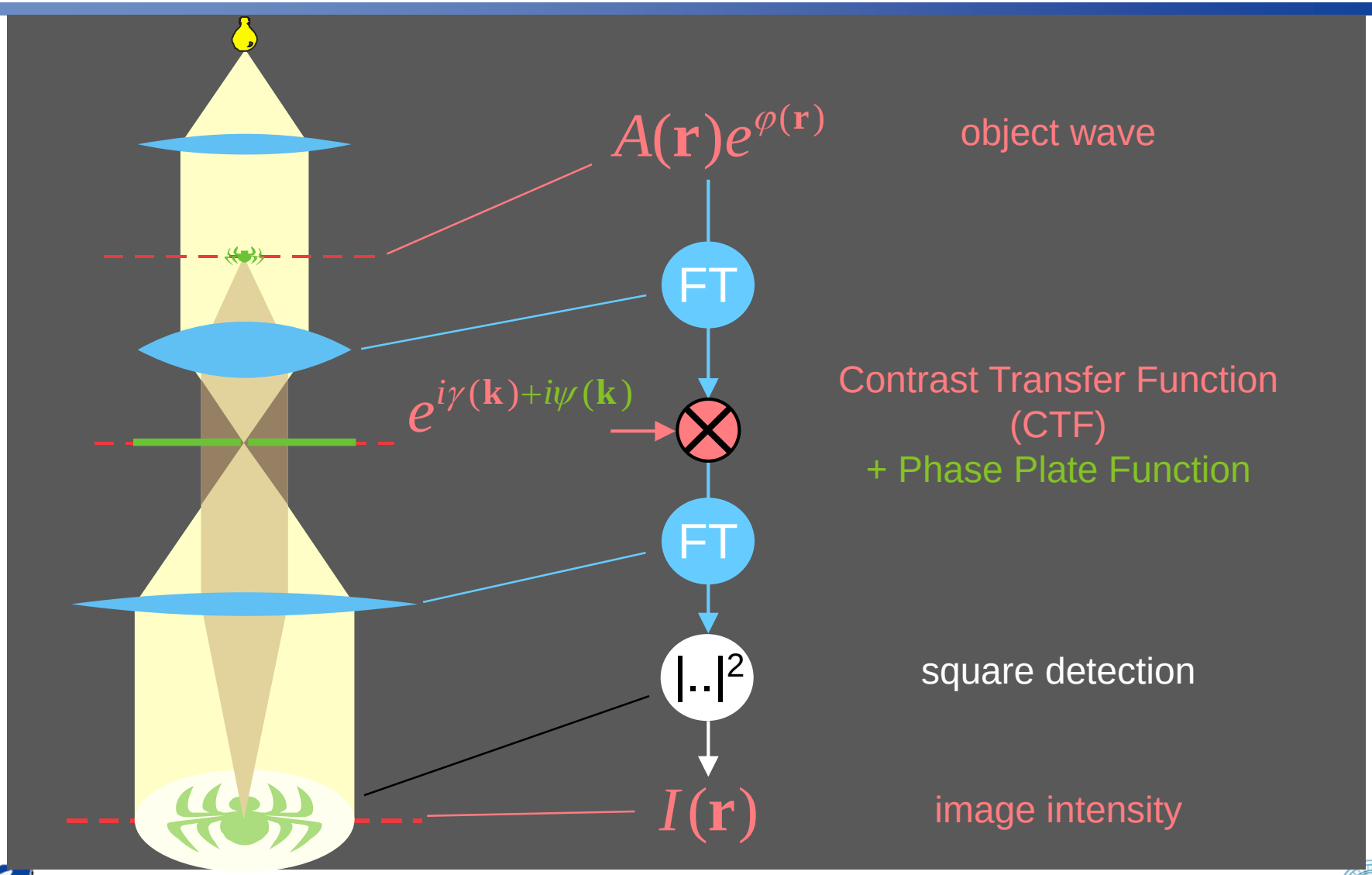
The Transmission Electron Microscope (TEM)



Mathematical model of TEM



Effect of the phase plate



Phase contrast transfer function – weak object

$$\mathrm{CTF}(k) = \sin\left[2\pi \underbrace{\left(-\frac{1}{2}\lambda\,\Delta z\,k^2 + \frac{1}{4}\lambda^3 C_s\,k^4 \right)}_{\gamma(k)} + \varphi \right]$$

aberration term

phase shift

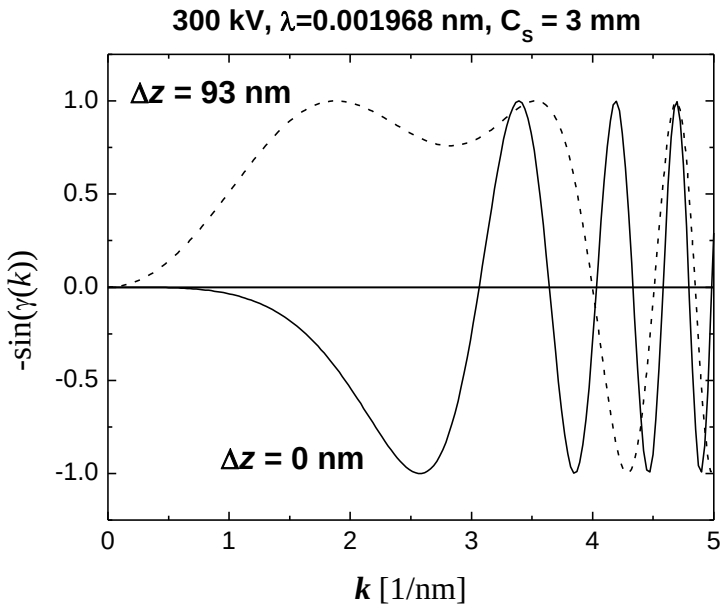
Ideal case – 90° phase shift

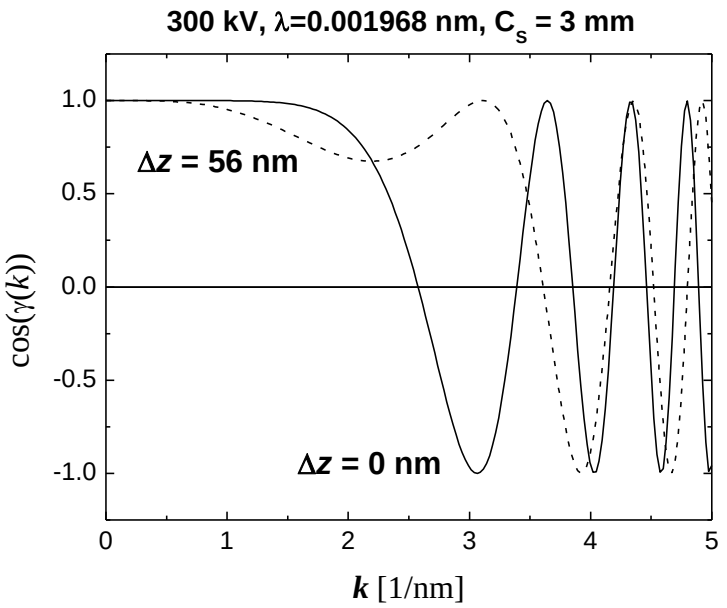
conventional TEM

phase plateTEM

$$\sin(\gamma(\mathbf{k}))$$

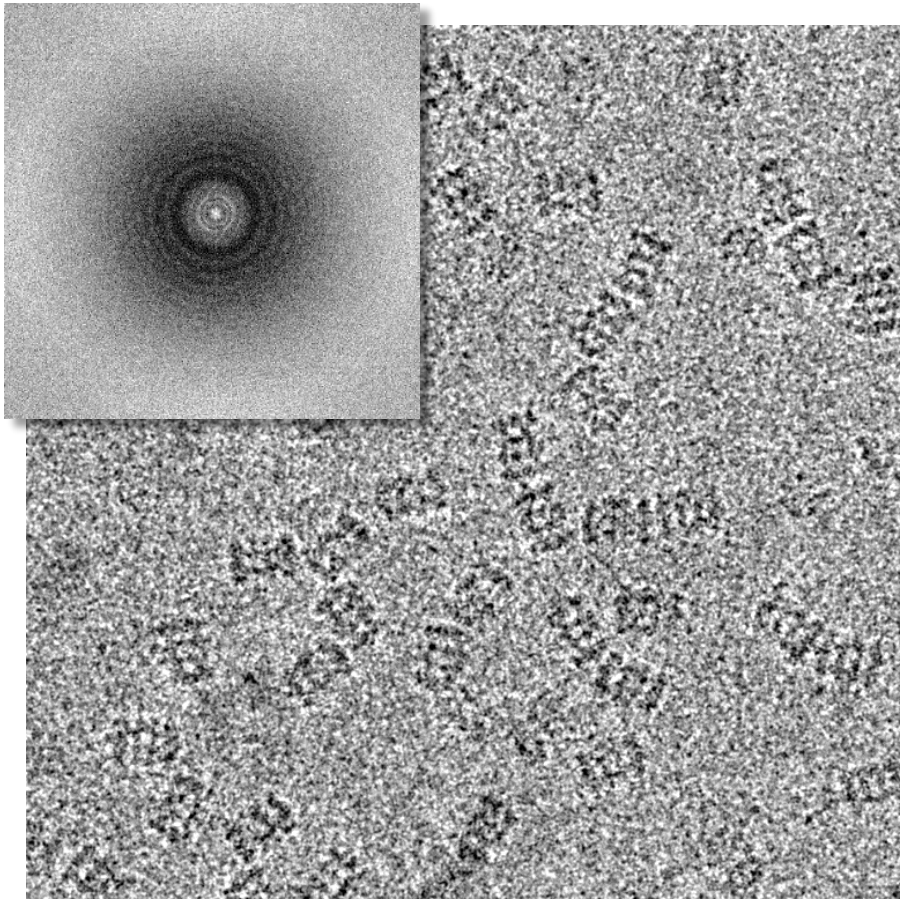
$$\cos(\gamma(\mathbf{k}))$$



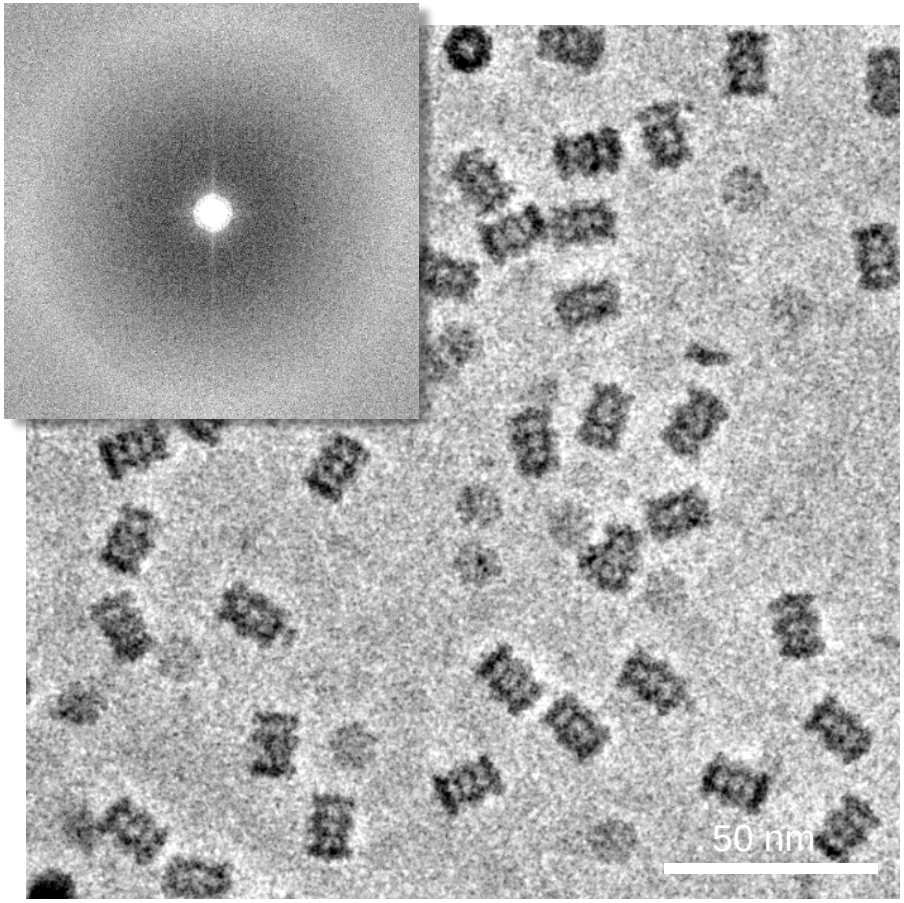


Phase plates improve the contrast

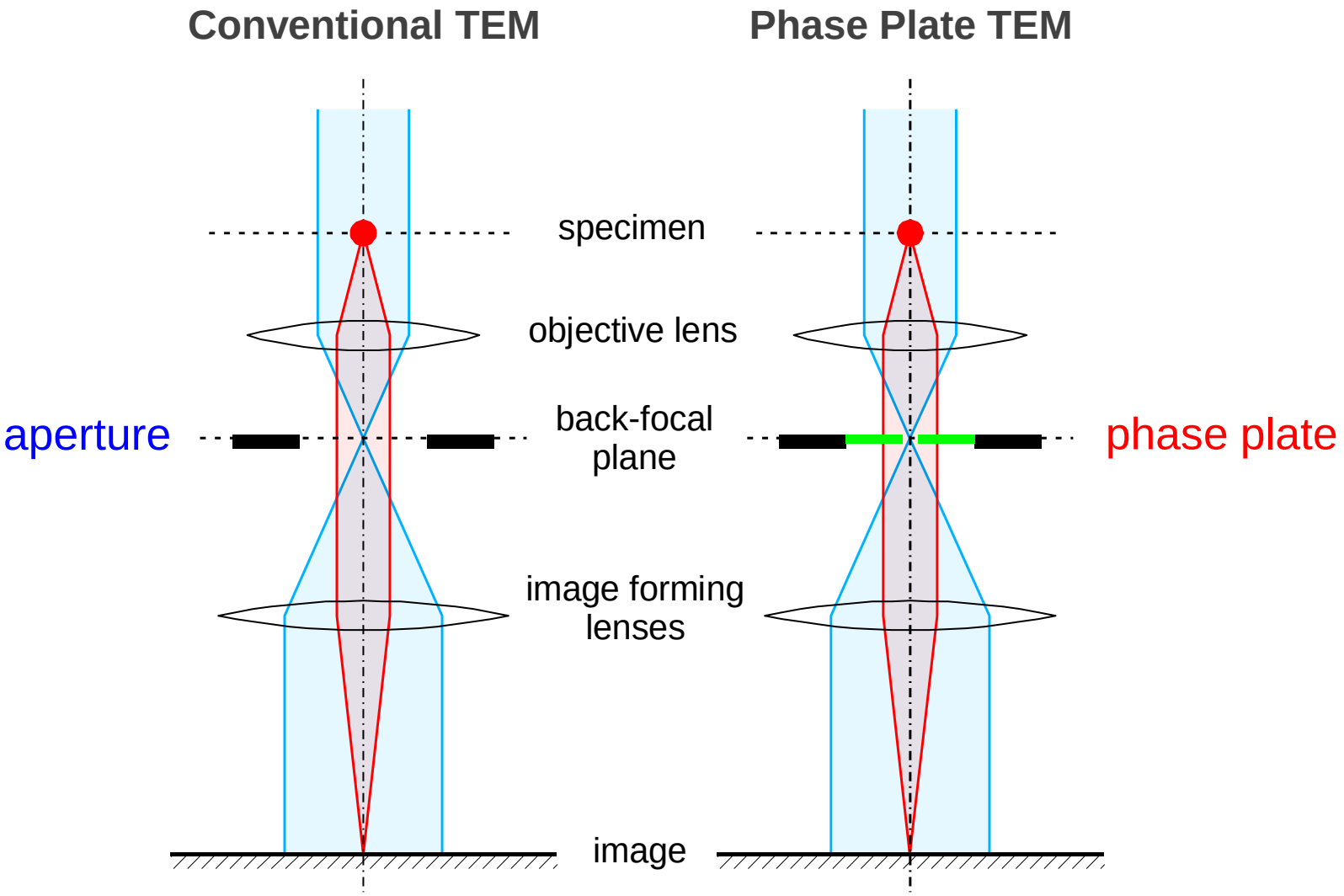
Conventional cryo-EM
1.5 um defocus



VPP cryo-EM
in-focus



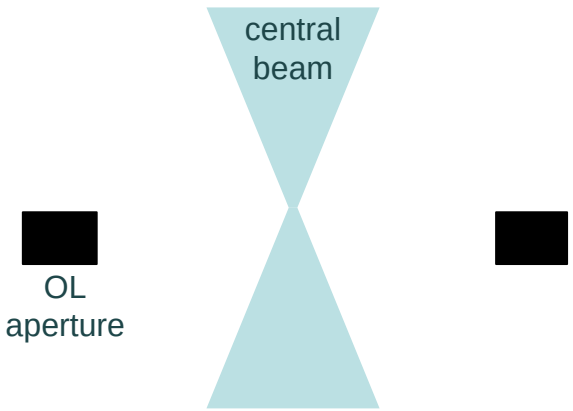
TEM imaging modes



TEM imaging modes

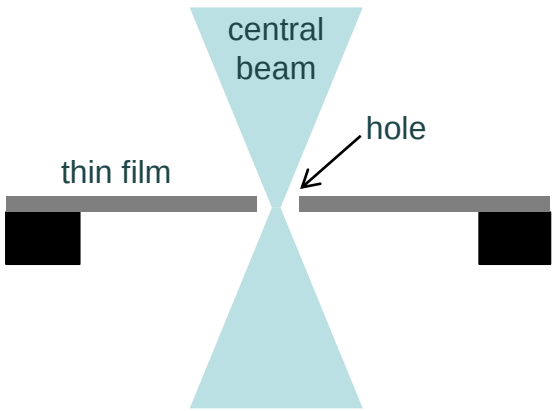
Conventional TEM

CTEM



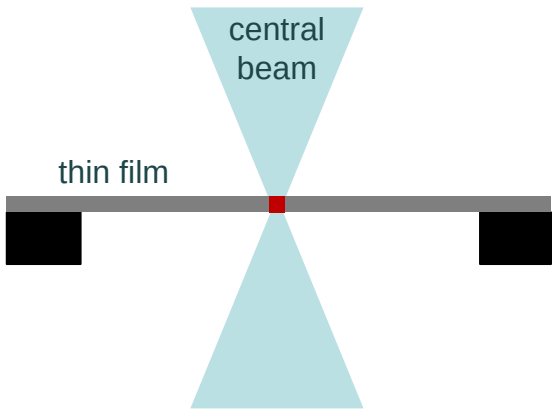
Zernike Phase Plate

ZPP



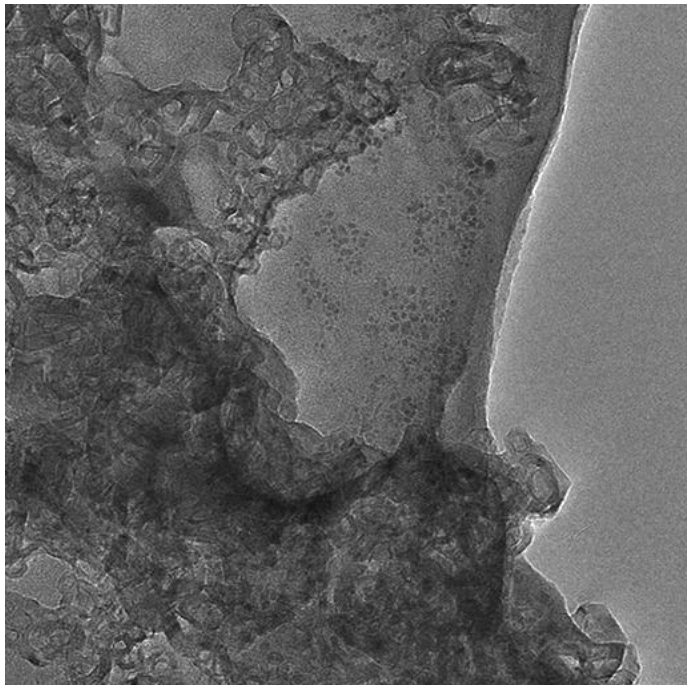
Volta Phase Plate

VPP

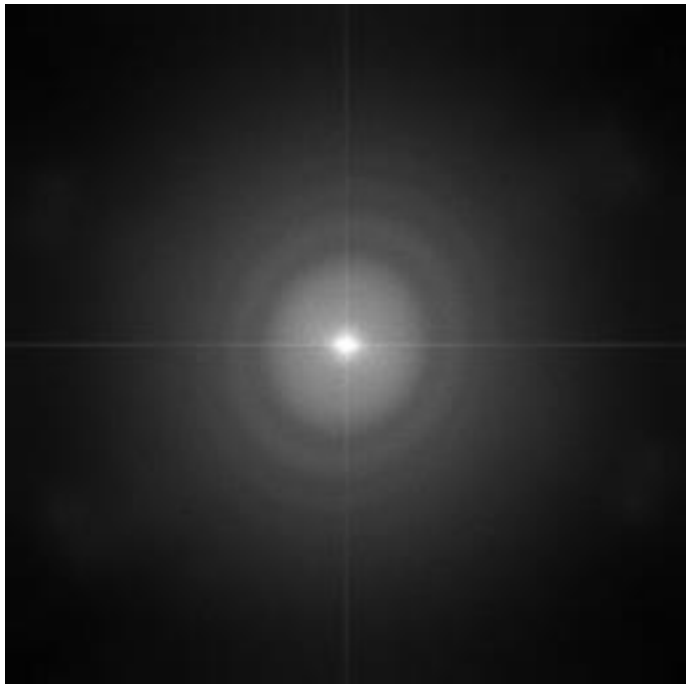


Volta phase plate - phase shift evolution

image series



FFT

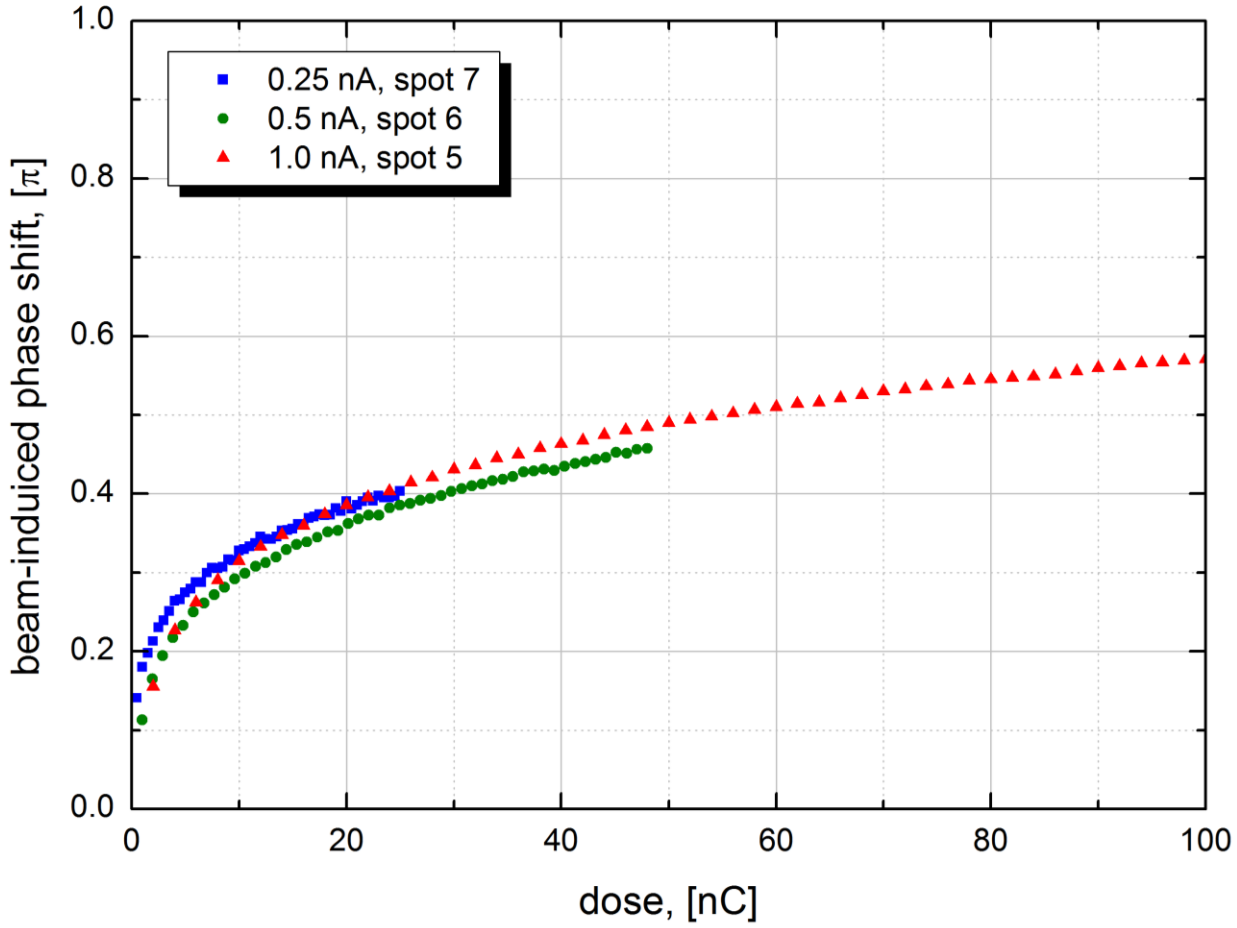


Niquist frequency ~ 4.3A

VPP – phase shift vs. beam current

- Danev *et al.*, "Volta potential phase plate for in-focus phase contrast transmission electron microscopy", *PNAS, 2014*

- The phase shift depends on the total dose and not on the dose rate.



Bridget's questions

1. **Are phase plates a key to high resolution of small and heterogeneous particles?**

2. **Can we make them easier to use?**

3. **What are the remaining issues?**

4. **Will there be progress in the near future for these devices?**

Bridget's questions

1. **Are phase plates a key to high resolution of small and heterogeneous particles?**
2. **Can we make them easier to use?**
3. **What are the remaining issues?**
4. **Will there be progress in the near future for these devices?**

Theoretical estimates

- R. Henderson, “The potential and limitations of neutrons, electrons and x-rays for atomic-resolution microscopy of unstained biological molecules”, *Quarterly Reviews of Biophysics*, 1995

- The **smallest particle size** for which the orientations can be determined is approximately **38/C^2 kDa**, where $0 < C \leq 1$ is the contrast relative to that of a perfect phase contrast image.

- The **number of particles** required is **38,000/*d***, where *d* is the resolution in Å.

Simulated ZPP data – size test

- Chang *et al.,* "Zernike phase plate cryoelectron microscopy facilitates single particle analysis of unstained asymmetric protein complexes", *Structure*, 2010

Table 1. Contrast of Simulated cryo-EM Images

	Diameter (nm)	Ideal (lossless)	ZEM (lossless)	ZEM (30% loss)	Defocus (μm) (CEM) 0.25	0.5	1	2	3	5
GroEl (840 kDa)	14	0.130	0.123	0.110	0.010	0.013	0.018	0.029	0.031	0.045
pol II (500 kDa)	13	0.125	0.111	0.095	0.017	0.022	0.026	0.033	0.036	0.038
TfR (290 kDa)	10	0.112	0.098	0.082	0.016	0.020	0.024	0.029	0.033	0.036
T7 pol-lys (100 kDa)	8	0.094	0.078	0.065	0.013	0.017	0.017	0.029	0.030	0.030

Cut-on frequency for ideal EM is 0 nm^{-1} and for ZEM is 0.083 nm^{-1}. GroEl was simulated by 300 kV (for ZEM with loss: 20%); others by 200 kV (for ZEM with loss: 30%). See also Table S1.

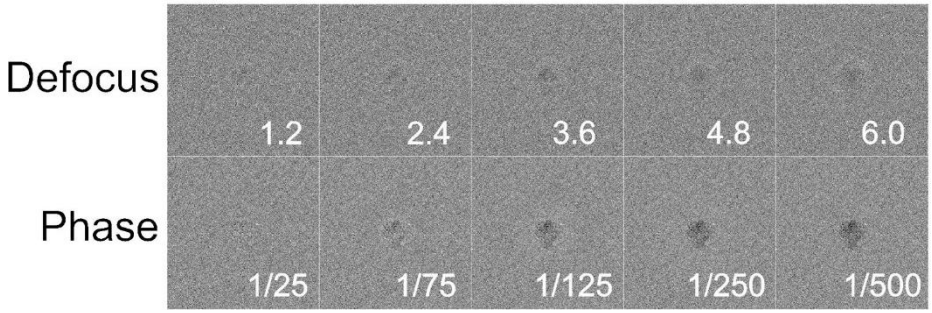
Table 2. Total Number of Particles Required to Reach a Target Resolution

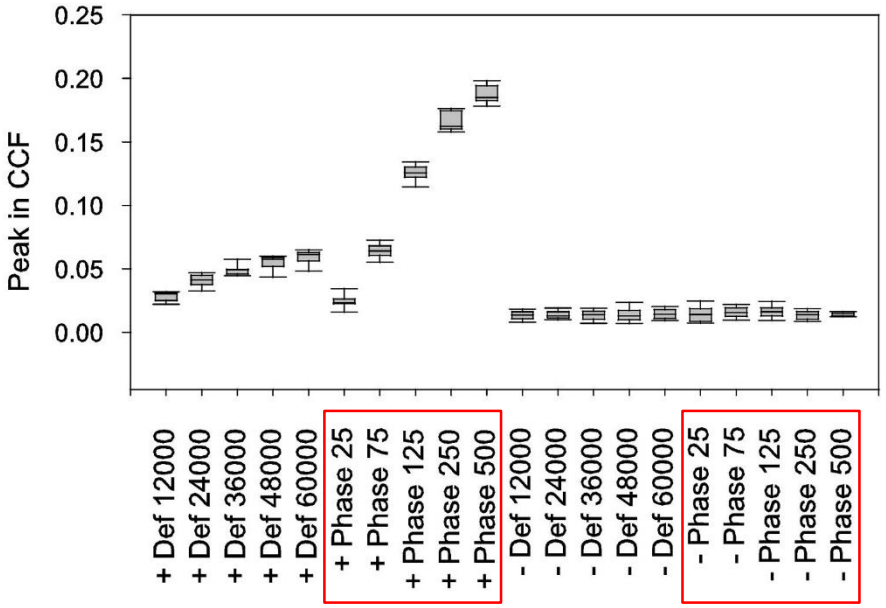
	Resolution (Å)	Ideal	ZEM (30% loss)	Defocus (μm) (CEM) 0.25	0.5	1	2
pol II	11	~800	~1,570	NA	~3,200	~2,200	~3,200
	4.5	~4,800	~15,000	<500,000	~38,000	~48,000	~240,000
500 kDa	3.3	~13,000	~60,000	<500,000	~260,000	~400,000	>500,000
TfR	11	~800	~1,400	NA	NA	~3,900	~6,400
	4.5	~5,000	~16,000	NA	NA	>600,000	>600,000
290 kDa	3.3	~21,000	~48,000	NA	NA	>1,000,000	>1,000,000
T7 pol-lys	11	~4,000	~6,400	NA	NA	~63,000	~160,000
	4.5	~21,000	~62,000	NA	NA	>1,000,000	>1,000,000
100 kDa	3.3	~66,000	~160,000	NA	NA	>1,000,000	>1,000,000

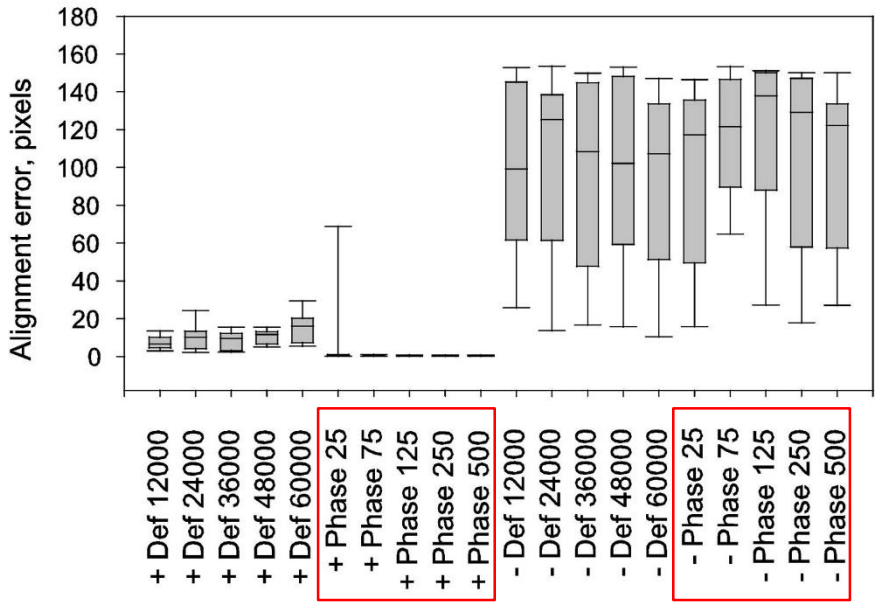
Simulated ZPP data – size test

- Hall *et al.*,"Accurate modeling of single-particle cryo-EM images quantitates the benefits expected from using Zernike phase contrast", *Journal of Structural Biology*, 2011

100 kDa protein



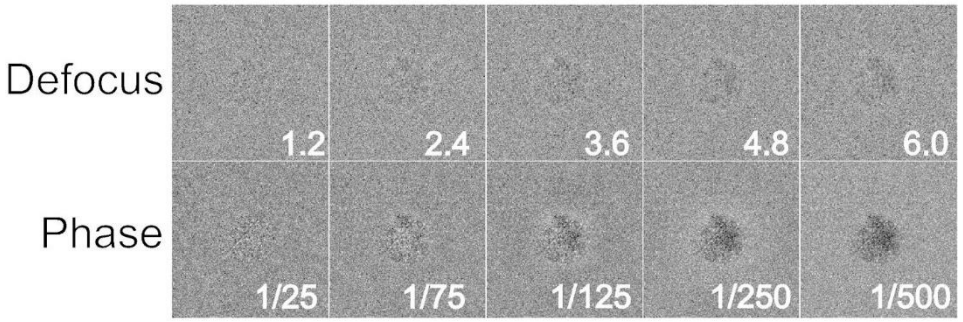


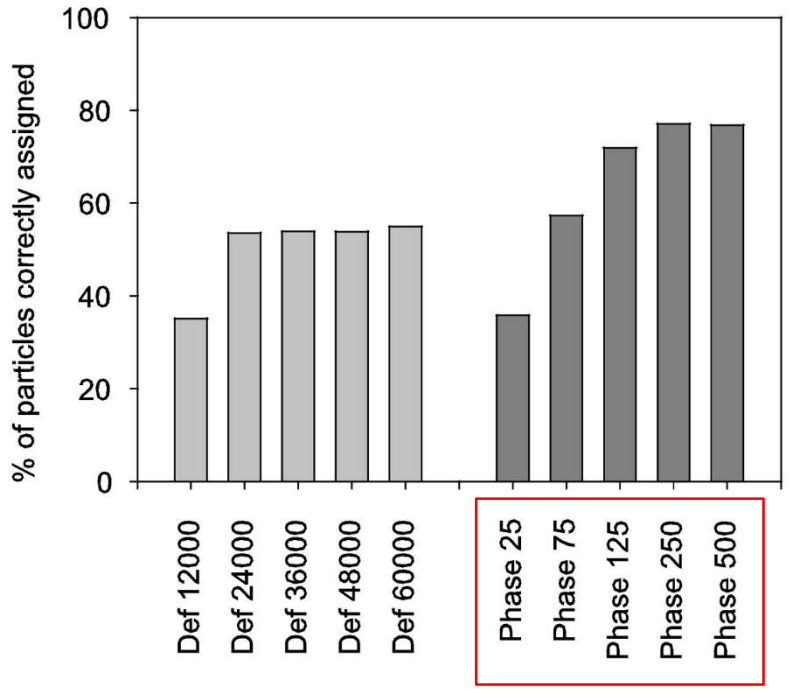


Simulated ZPP data – heterogeneity test

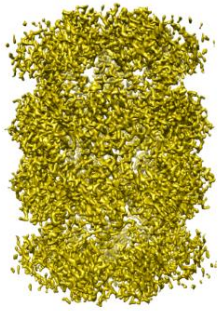
- Hall *et al.*,"Accurate modeling of single-particle cryo-EM images quantitates the benefits expected from using Zernike phase contrast", *Journal of Structural Biology*, 2011

~200 kDa protein
+- 12.5 kDa
+- 33 kDa



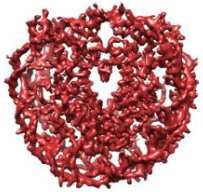


Structures solved with the VPP

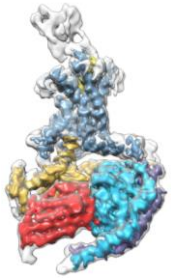


20S proteasome
700 kDa, 2.4 Å
EMD-3455

Peroxiredoxin-3
257 kDa, 4.4 Å
EMD-3233
Preferred orientation, thick ice

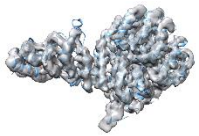


Nucleosome
200 kDa, 3.9 Å
EMD-8140
Smallish size, low contrast in top views

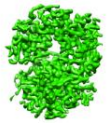


Calcitonin GPCR
150 kDa, 4.1 Å
EMD-8623
Smallish size, some flexibility

GLP-1 GPCR
150 kDa, 3.3 Å
EMD-7039
Smallish size



Rpn1
100 kDa, 3.8 Å
Small size, preferred orientation



Hemoglobin
64 kDa, 3.2 Å
EMD-3488
Small size

Answer to the 1st question

1. **Are phase plates a key to high resolution of small and heterogeneous particles?**

- They seem to help < 200 kDa.
- They may be the key to many < 100 kDa structures.
- Heterogeneity is too complex to generally quantify, but phase plates are expected to improve the performance of classification.
- Previous image simulations were done before “the revolution” without taking into account new methods, such as frame alignment and dose weighting.
- Frame alignment should benefit from the use of a phase plate by using a finer temporal sampling, i.e. “super fractionation”.
- Dose weighting improves greatly the performance of the conventional defocus approach for small particles.
- We need up-to-date image simulations!

Bridget's questions

1. Are phase plates a key to high resolution of small and heterogeneous particles?

2. **Can we make them easier to use?**

3. What are the remaining issues?

4. Will there be progress in the near future for these devices?

VPP alignments - on-plane condition

Volta Phase Plate

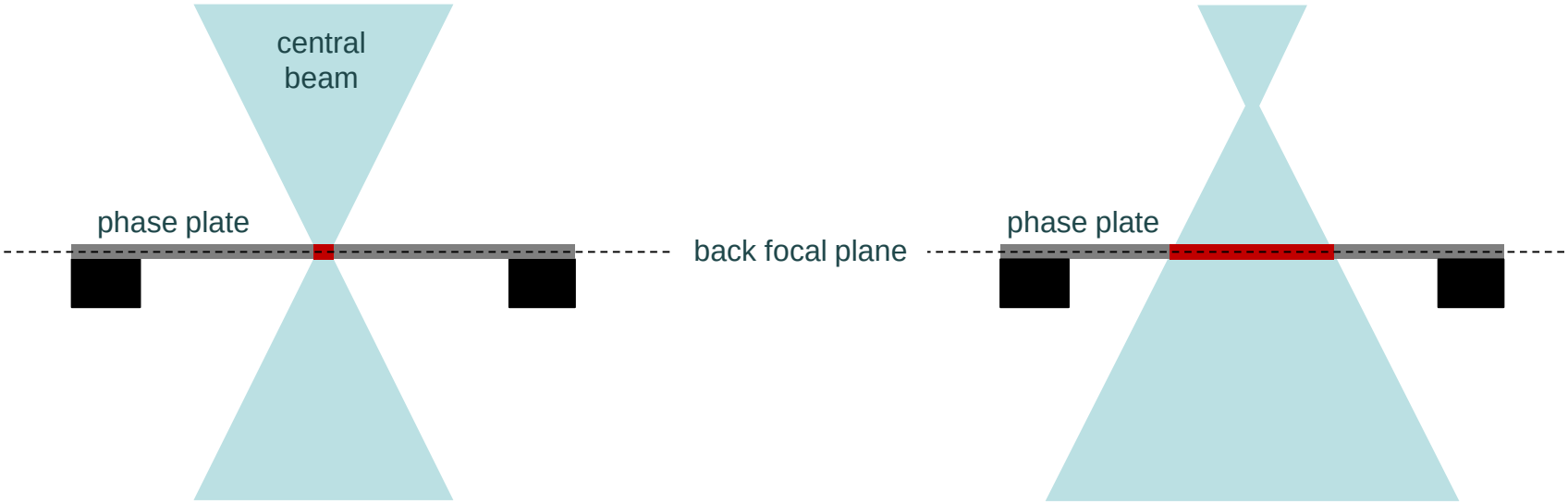
on-plane

(parallel illumination)

Volta Phase Plate

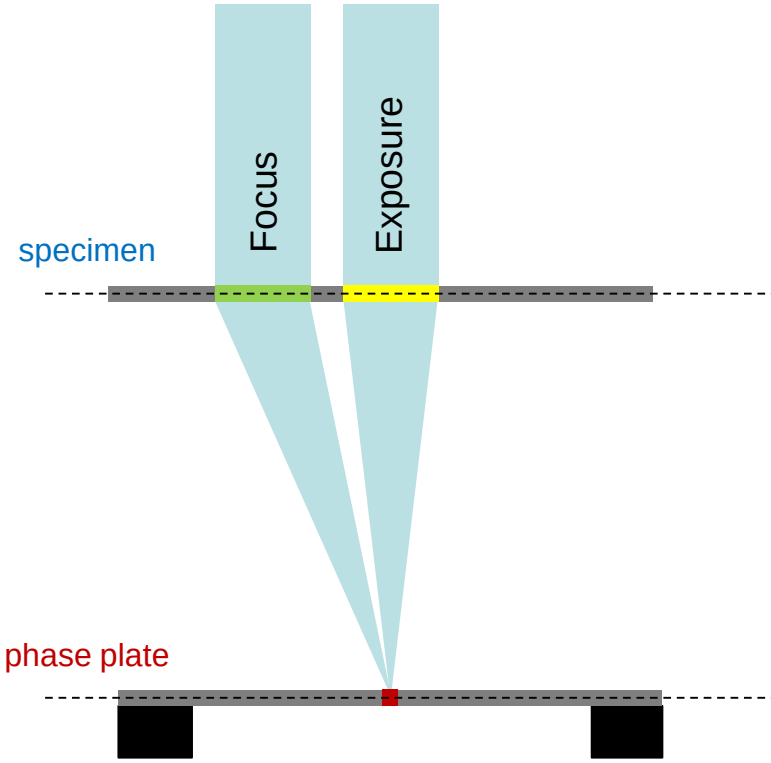
off-plane

(non-parallel illumination)

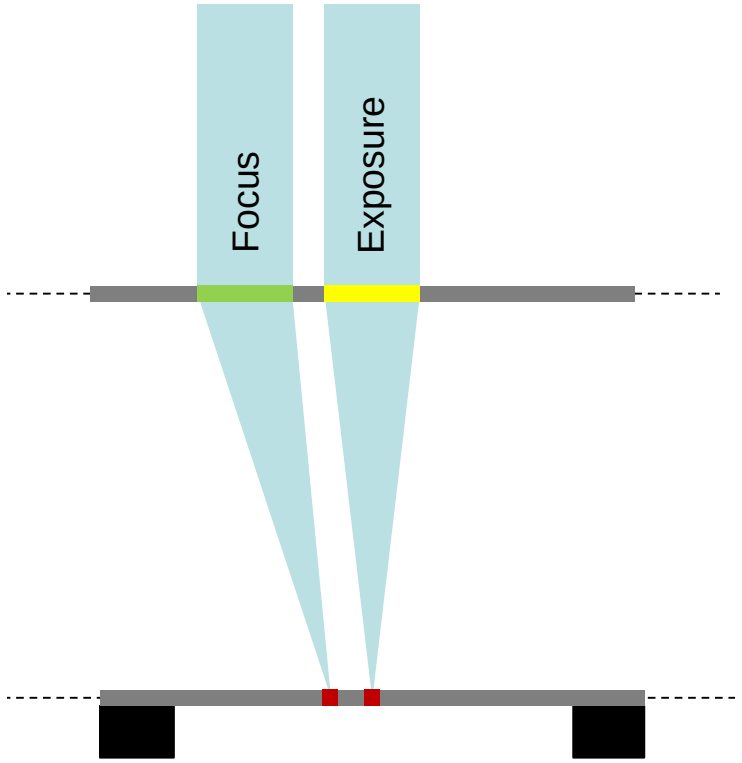


VPP alignments - beam shift pivot points

Correct pivot point setting

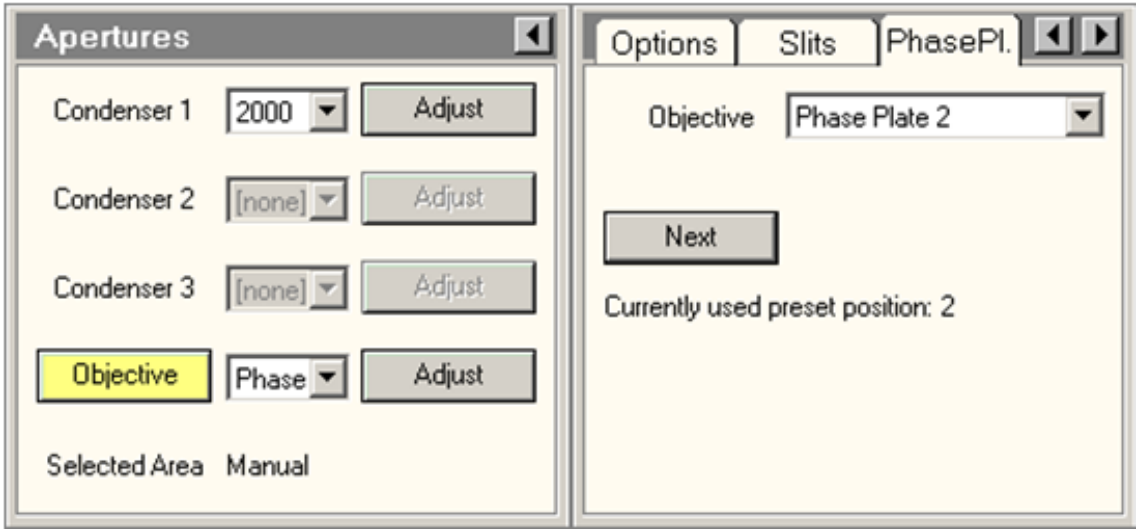


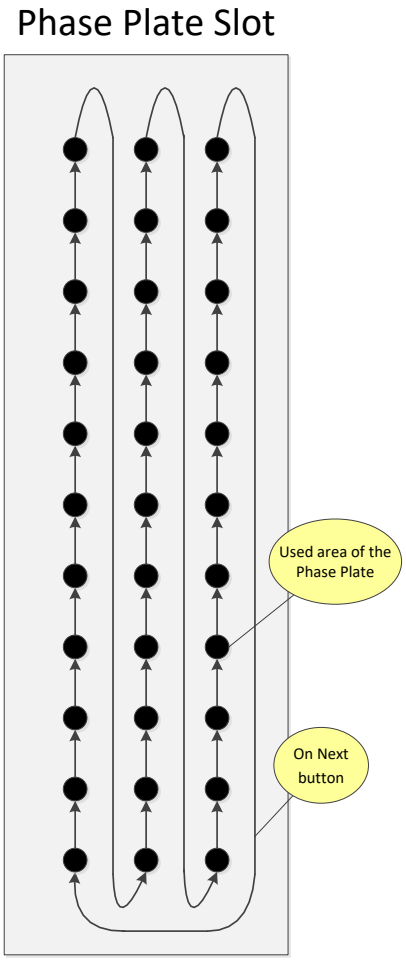
Incorrect pivot point setting



FEI tools for phase plate navigation

- 6 slots x 76 positions → 456 fresh areas
- Single area for ~1 hr operation





Answer to the 2nd question

2. Can we make them easier to use?

- Using preset imaging states which store all alignments could greatly simplify their use.
- An automated phase plate quality test/enumeration could make the VPP simpler to use by only allowing access to good phase plate positions.
- Track the usage of each VPP position and go to positions which have had the longest time to recover?
- Future phase plates (laser) may be easier to use in some ways and more difficult in others.

Bridget's questions

1. Are phase plates a key to high resolution of small and heterogeneous particles?

2. Can we make them easier to use?

3. **What are the remaining issues?**

4. Will there be progress in the near future for these devices?

Volta phase plate issues

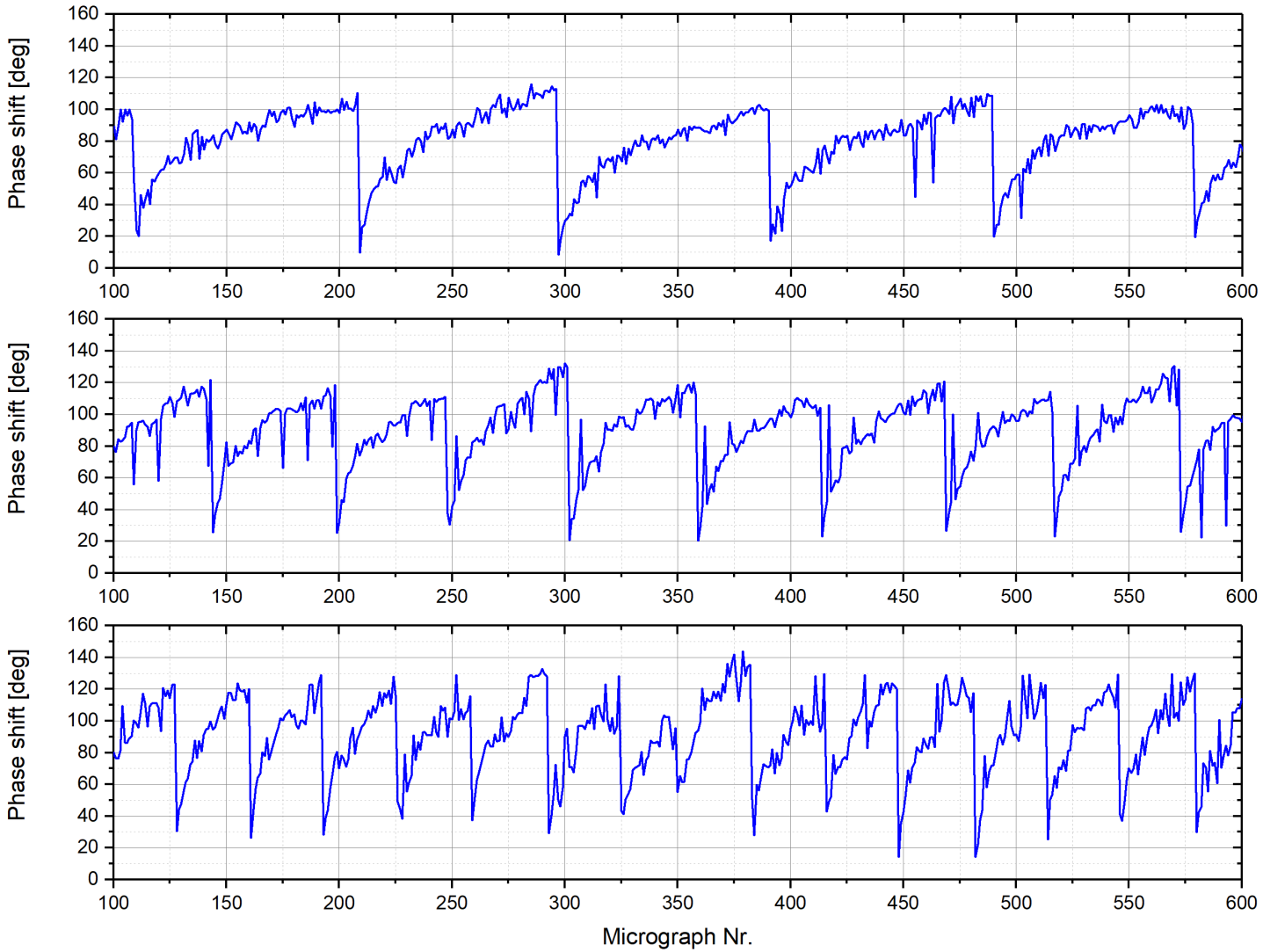
1. **Inconsistency in phase plate behavior.**
   - There are “fast” and “slow” phase plates, i.e. different labs observe different phase shift development speeds.
   - The VPP seems to “age” in terms of phase shift speed evolution.
   - Old VPP spots may recover very slow or not recover completely (“scars”).
2. **Methodological issues.**
   - Inaccurate alignments – save and load imaging states.
   - “Focus spots” disturbing nearby positions on the VPP – change tilt direction.
3. **Intrinsic issues.**
   - Information loss of ~18% @ 200 kV, ~15% @ 300 kV – make it thinner.
   - Additional astigmatism of up to ~1000 Å which varies depending on the position on the VPP.
   - Variable phase shift complicates the CTF fitting.
   - Gets dirty over time (years).
   - “Blown away” phase plates.

Volta phase plate “maturation”

1 day old VPP
94 mics/position

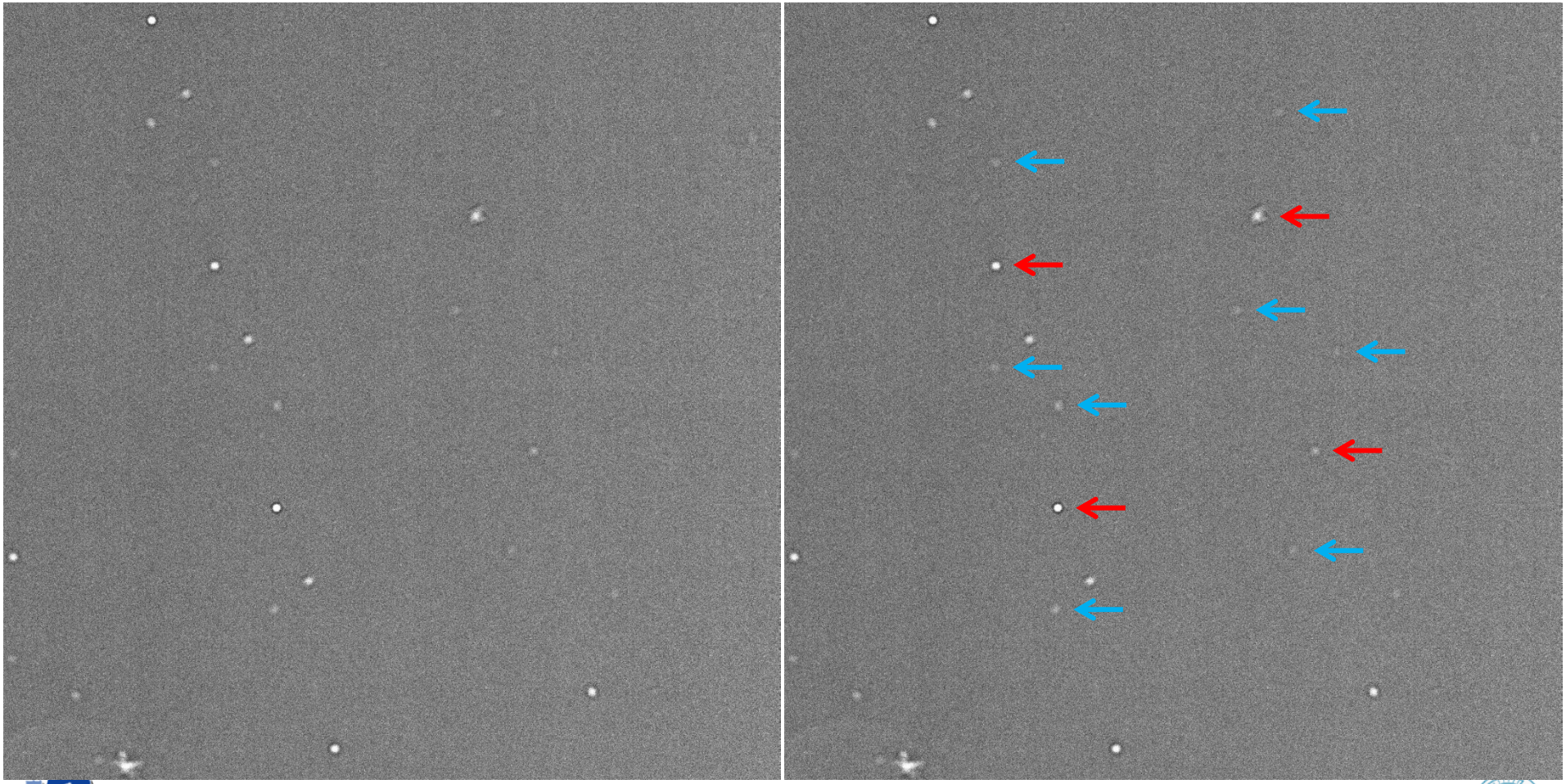
3 months old VPP
54 mics/position

7 months old VPP
32 mics/position

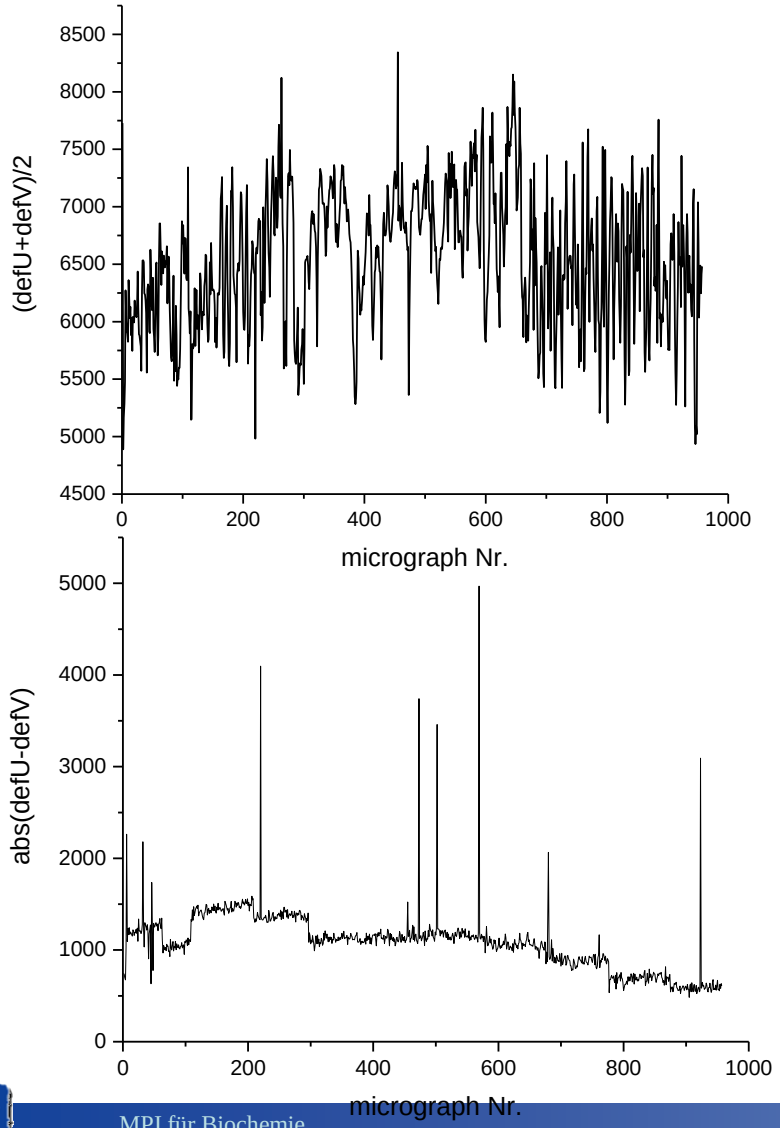


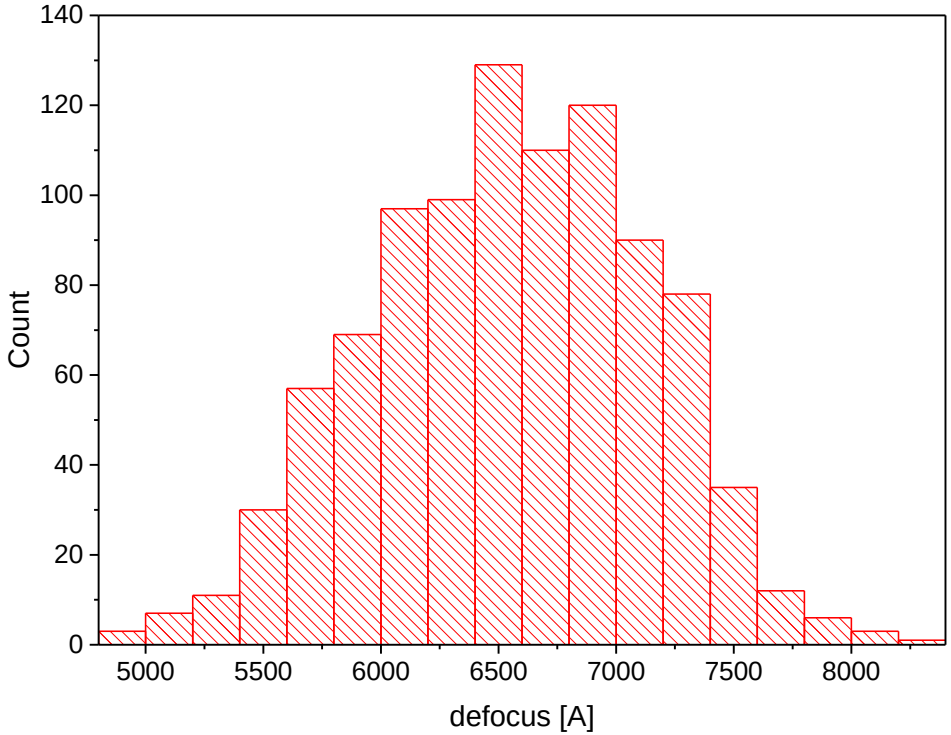
“Focus spots”

- 5 mrad beam tilt. Images provided by Mazdak Radjainia, Thermo Fisher

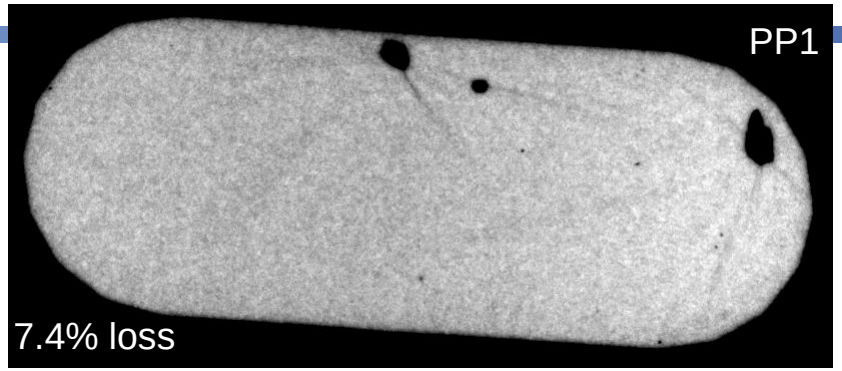


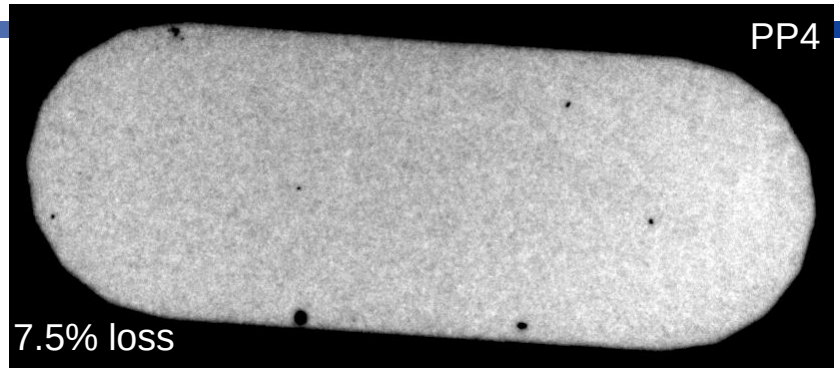
Gctf CTF fitting – defocus plots

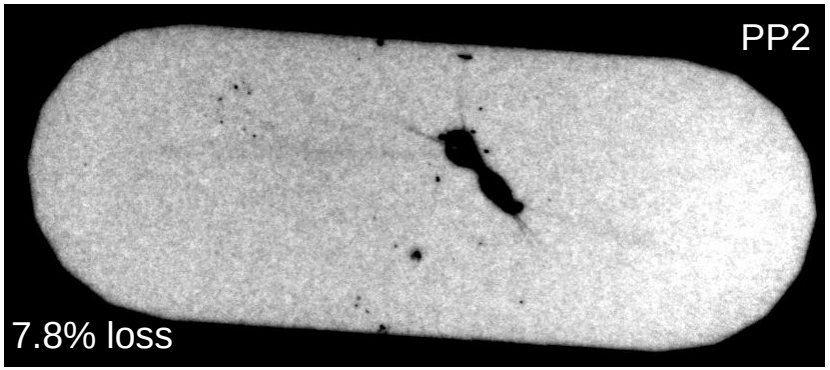


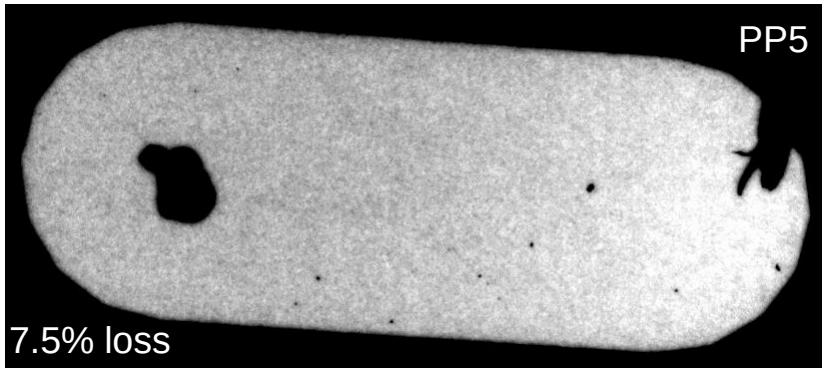


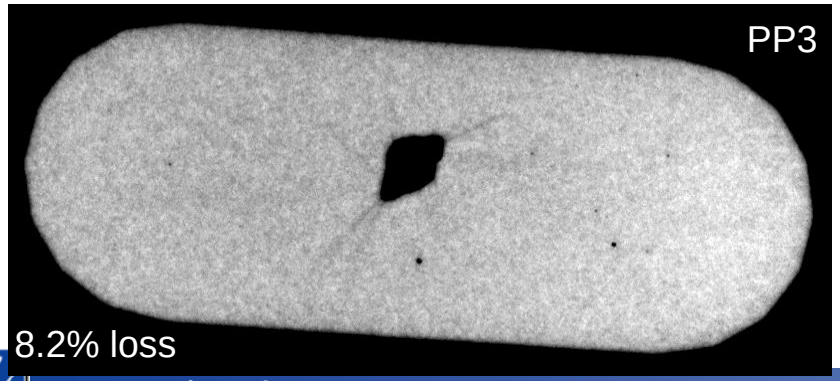
VPP condition after > 2 years in the microscope

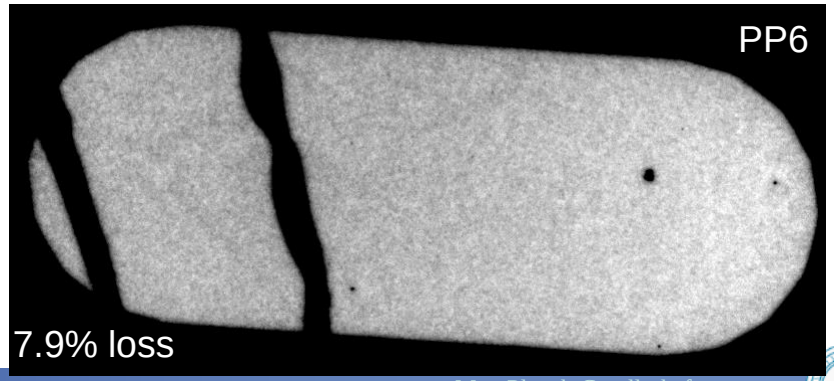












Bridget's questions

1. Are phase plates a key to high resolution of small and heterogeneous particles?

2. Can we make them easier to use?

3. What are the remaining issues?

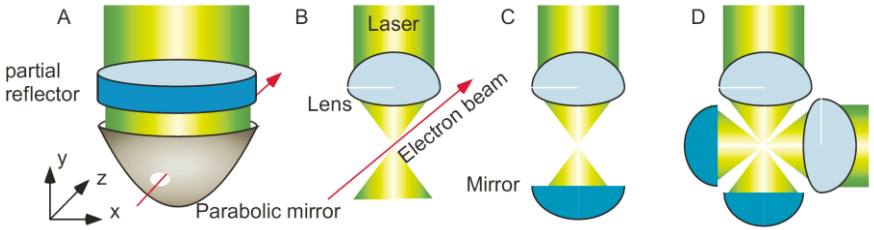
4. **Will there be progress in the near future for these devices?**

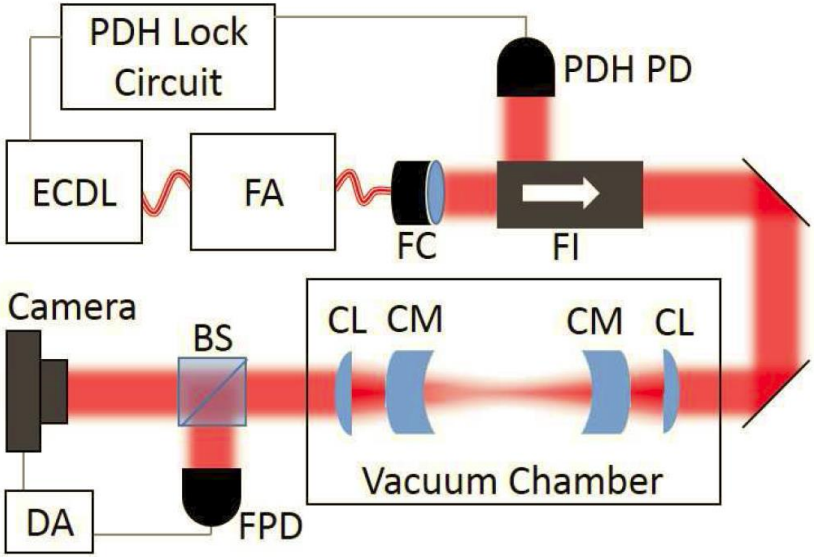
VPP improvements

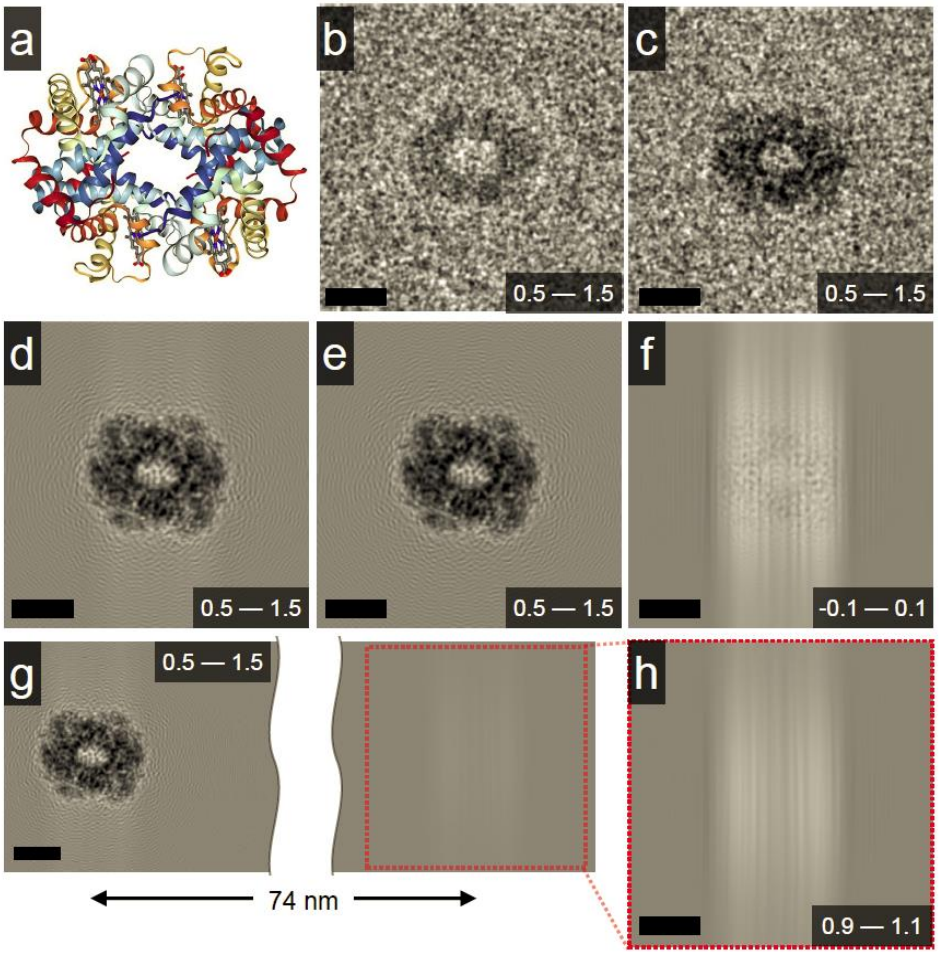
1. **Make it thinner.**
    - If we make it half as thick (5 nm) the information loss will be reduced approximately in half (~8%).
    - Try graphene.
    - Increasing the acceleration voltage from 300 kV to 1 MV will reduce the information loss by ~1/3.
2. **Improve the software.**
    - Enumeration, usage logs, etc.
3. **Make it more consistent.**
    - Improving the manufacturing would be quite difficult but it is not impossible.

Laser phase plate

- H. Müller *et al.*,"Design of an electron microscope phase plate using a focused continuous-wave laser", *New J. Phys.*, 2010
- O. Schwartz *et al.*,"Near-concentric Fabry-Pérot cavity for continuous-wave laser control of electron waves", Optics Express, 2017







The Volta phase plate for cryo-tomography

- **In my current opinion, it only makes sense to do in-focus tomography with the VPP.**
  - We tried VPP tomo with defocus but it requires similar amounts of defocus (> 3 um) as conventional acquisition. The SNR of the tilt images is much lower than single particle images which limits the ability to fit the CTF at lower defocuses.

- **The performance is limited by the ability to focus accurately and maintain the phase shift.**
  - Accurate beam-shift pivot points alignment is crucial!
  - Flat samples, such as in-vitro or thin cells on carbon, work well.
  - Cryo-FIB lamellas are quite tricky due to electrostatic charging of the sample and lamella pretilt. The success rate is < 30%.

Current VPP limitations in tomography

- Phase shift fluctuations due to beam movement on the VPP:
  - specimen charging
  - beam-shift pivot points not aligned properly
  - lens hysteresis – switching to View (Search) mode during the tilt series
  - other causes – normalizations, beam blanker/shutter quirks, magnetic parts in the goniometer etc.

- Accurate focusing:
  - the acquisition and tracking/focusing areas are not at the same Z-height
  - use three image focusing (drift protection) with zero defocus offset at each tilt

- Too much phase shift:
  - move the phase plate to a new position in the middle of the tilt series.

- Fukuda et al., “Electron cryotomography of vitrified cell with a Volta phase plate”, JSB 190 (2015).
- Khoshouei et al., “Subtomogram analysis using the Volta phase plate”, JSB (2016).
- Schaffer et al., “Optimized cryo-focused ion beam sample preparation aimed at in situ structural studies of membrane proteins”, JSB (2016).

The Volta Phase Plate for Single Particle Analysis

- **Initially we were using the VPP in-focus because of the lack of software support for phase plate CTF fitting and correction.**
  - The in-focus method is ideal from a theoretical point of view but is very cumbersome in practice.
  - Requires very accurate focusing and stigmation because such errors cannot be corrected during processing.
- **The VPP with defocus approach is much simpler and very similar to conventional defocus acquisition. We have been using this approach for the last 1.5 years.**
  - In practice, the optimal defocus is ~500 nm.
  - The applied defocus does not generate contrast, which is provided by the VPP, but enables accurate CTF fitting (> 5 CTF rings).

Summary of VPP for SPA

- Accurate CTF determination is very important!!!
  - 10 nm defocus error gives 90^{o} CTF phase shift at 2 Å periodicity: defocus refinement?
  - VPP phase shift error is not a big issue: the phase shift affects all frequencies equally.

- High phase shift (>0.7 π) images do not “behave” well, but so do low-phase shift ones.
  - prevent the phase shift from going too high by advancing the VPP more often.

- Optimal defocus.
  - no need to vary the defocus? The VPP phase shift evolution takes care of it?
  - optimal defocus ~ 500 nm. Take into account the offset due to Cs!
  - focus accurately using 3 image focusing and 0 defocus offset!
  - use 10 mrad beam tilt for focusing!

- Danev, R., Baumeister, W. Cryo-EM single particle analysis with the Volta phase plate. *eLife* 5, 2016
- Danev, R., Tegunov, D., Baumeister, W. Using the Volta phase plate with defocus for cryo-EM single particle analysis. *eLife* 6, 2017

Thank you for your attention!