Lecture 5

Cryo-electron microscopy

Tibor Füzik

“The structural biology continuum”

polypeptides

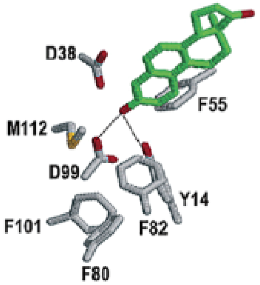
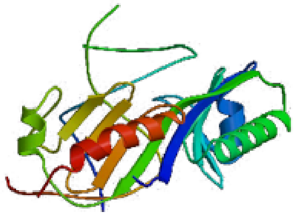


Figure 9 from Park and Merz,
JACS 125:901

molecular
dynamics
simulations

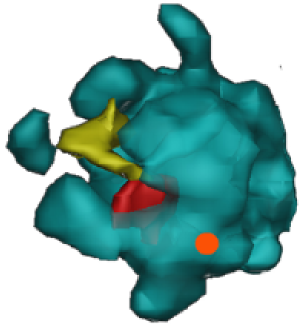
small proteins
and domains



Human TBP and DNA
Nikolov et al., PNAS 93:4862

X-ray crystallography
NMR spectroscopy

large proteins
and complexes



RNA Polymerase II

cryoEM single
particle analysis
or X-ray
crystallography

multi-protein
reactions

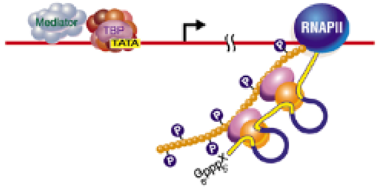


Figure 3 from Orphanides and Reinberg,
Cell 108:439

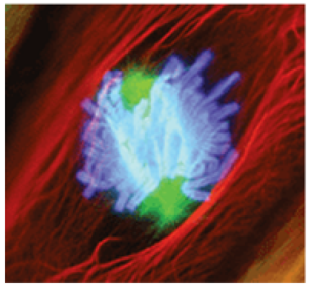
cryoelectron
tomography

whole cells
or cell sections

C. crescentus cell

cryoelectron
tomography,
light microscopy

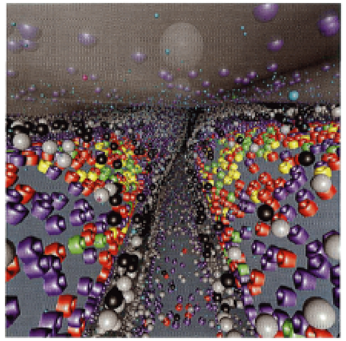
whole cells



Mitosis
Conly Reider and
Alexey Khodjakov
Science 300 #5616 cover

fluorescence
light
microscopy

whole cells
and tissues



Synapse
Stiles and Bartol,
Computational Neuroscience
CRC Press

structurally and
spatially explicit
cell modeling

Grant Jensen

Å nm µm mm

How does it compare to other methods

X-ray crystallography

NMR

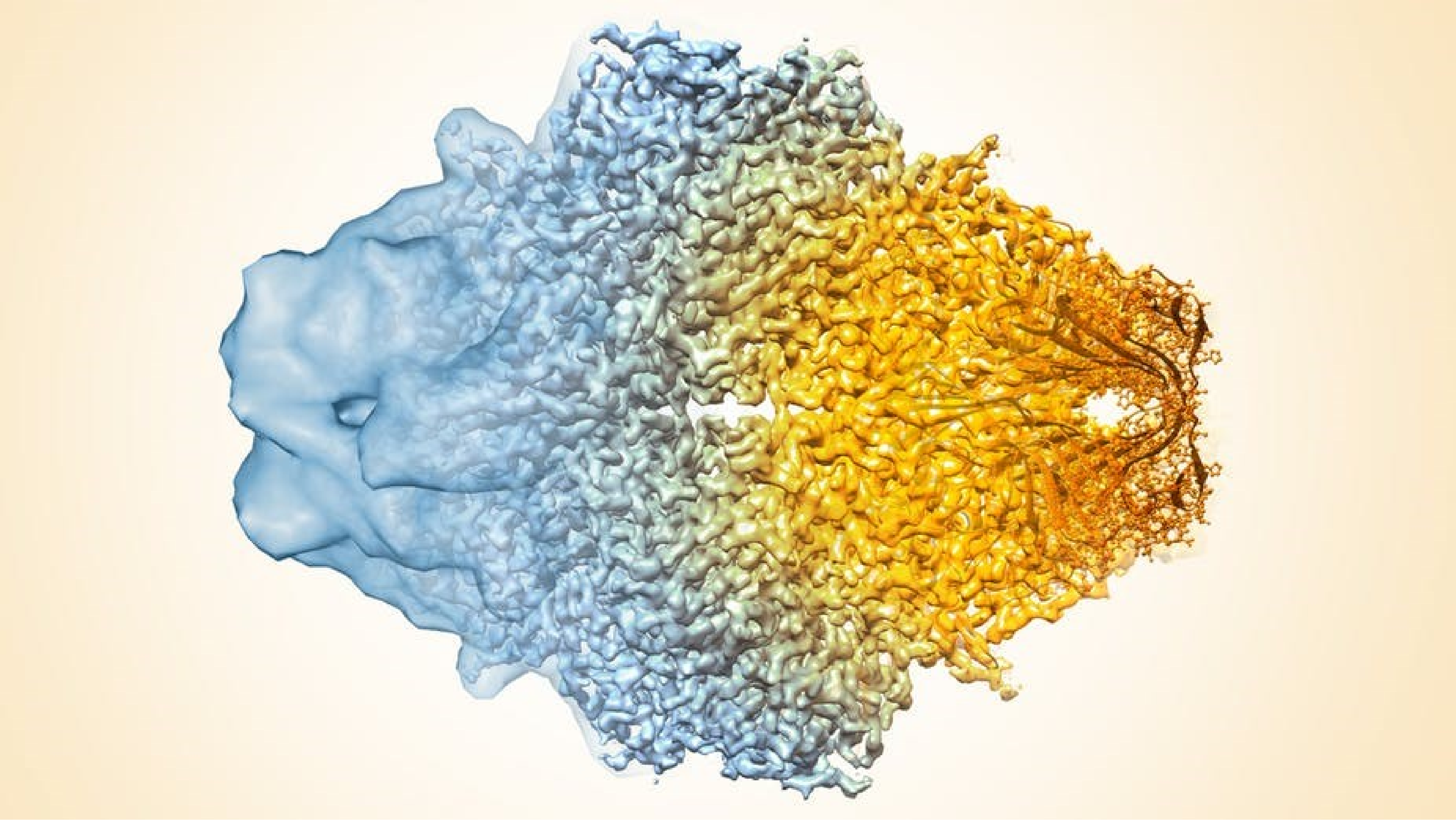
Cryo-EM

Nobel Prize in Chemistry 2017

Source: nobelprize.org

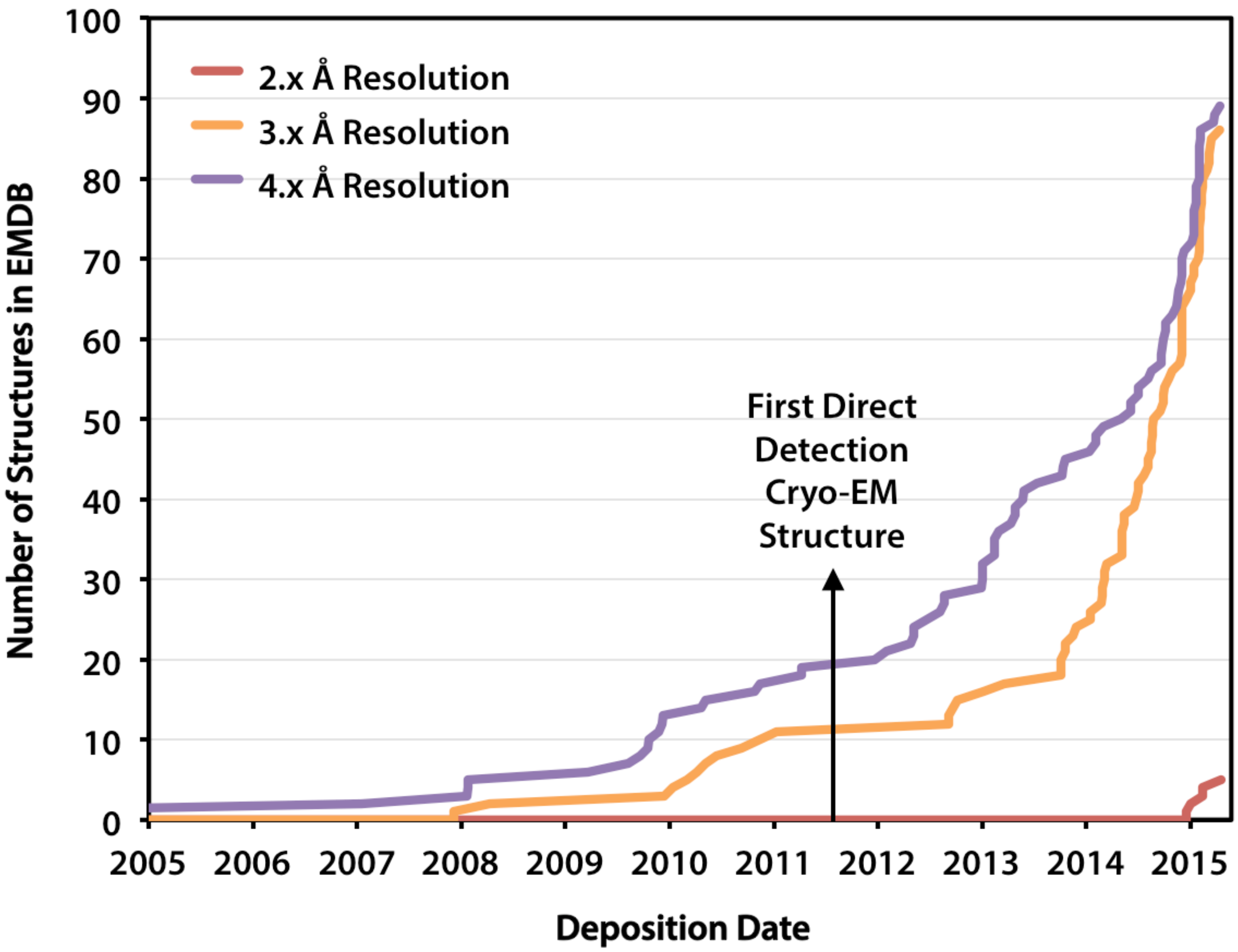
© DW

The resolution revolution



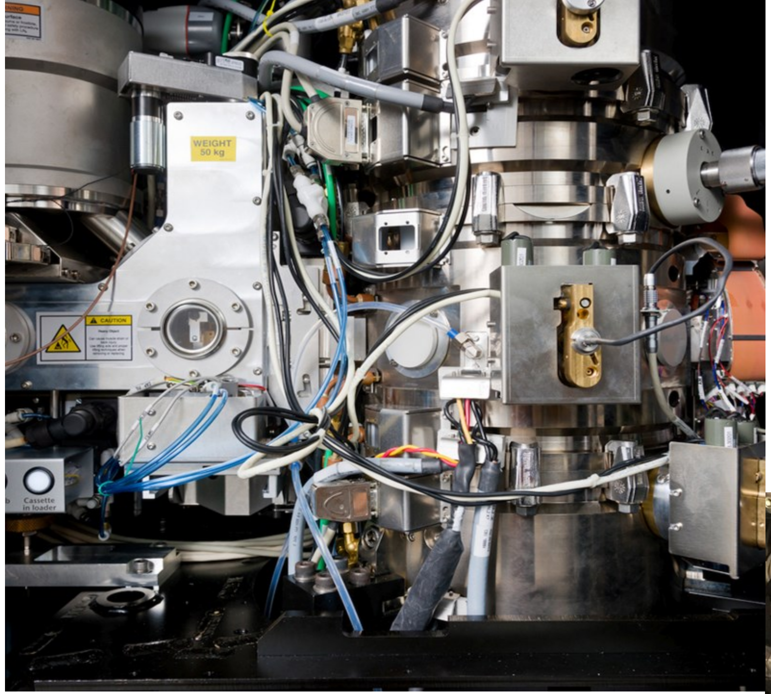
beta-galactosidase; Veronica Falconieri, Sriram Subramaniam, National Cancer Institute

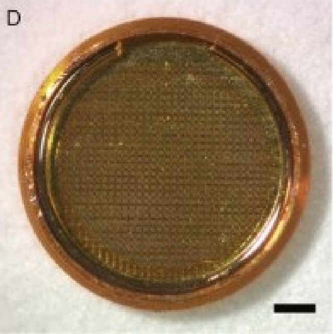
The resolution revolution

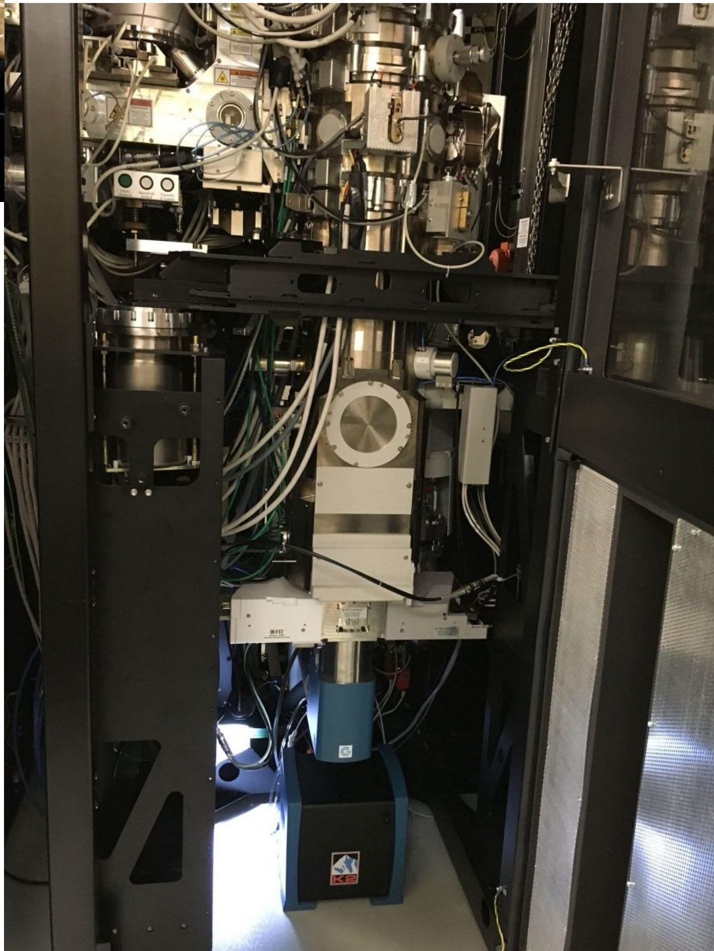


The Microscope

Titan KRIOS





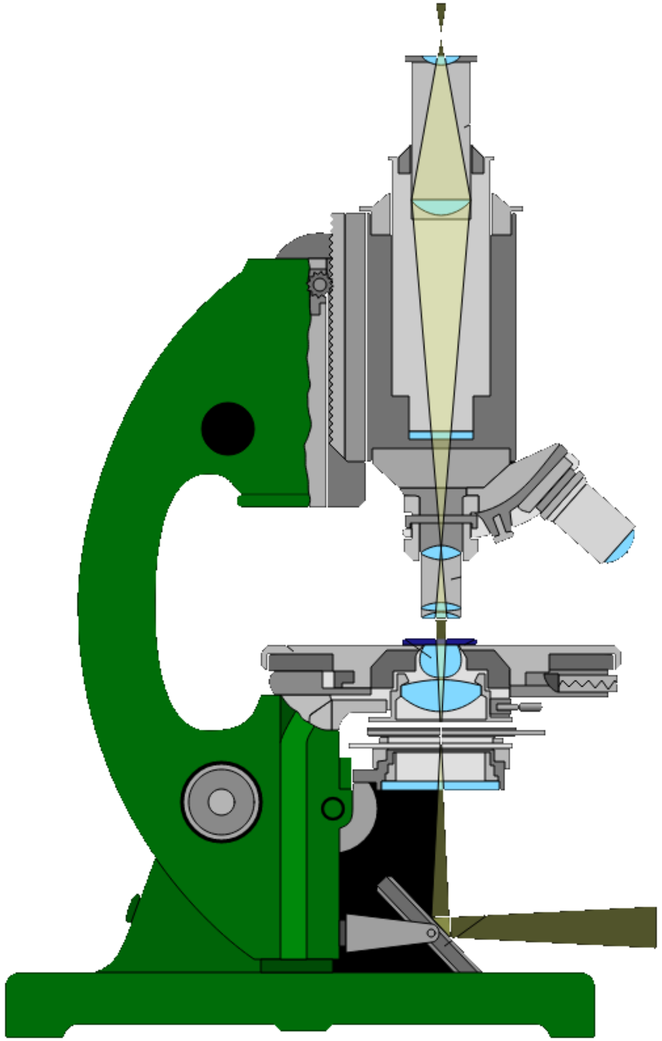


- X-FEG – 300 kV
- 3 condenser illumination system
- no Cs corrector
- autoloader for 12 samples
- cooled to liquid N_2 temperature
- energy filter
- CCD, DED

How TEM compares to optical microscope

Transmission electron microscope

Optical microscope



Source of electrons

Illumination system

Sample (electron transparent)

Objective lens

Projection system

Detector

Detector

Projection system

Objective lens

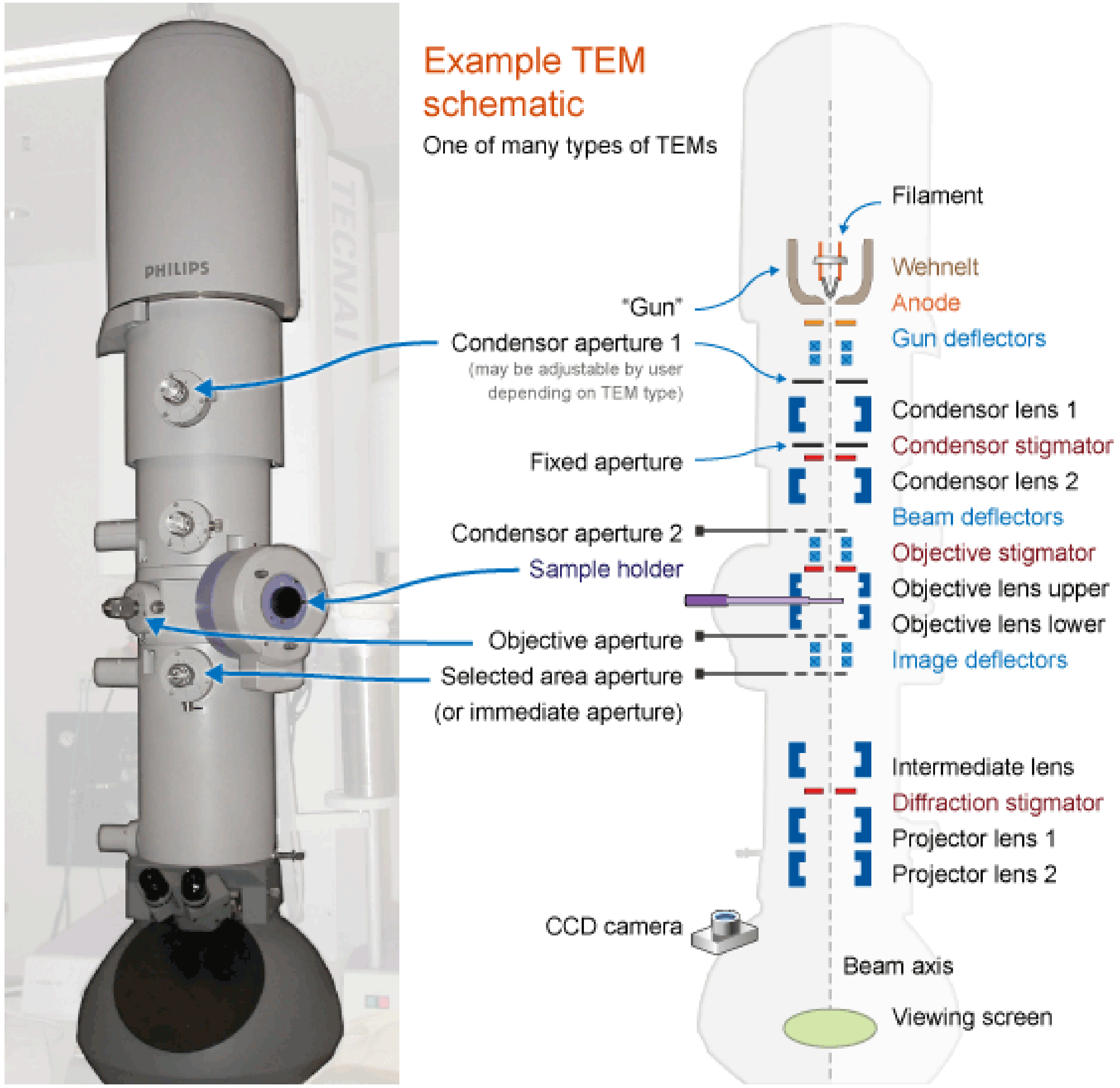
Sample (transparent)

Illumination system

Source of photons

bio.libretexts.org

TEM schematic

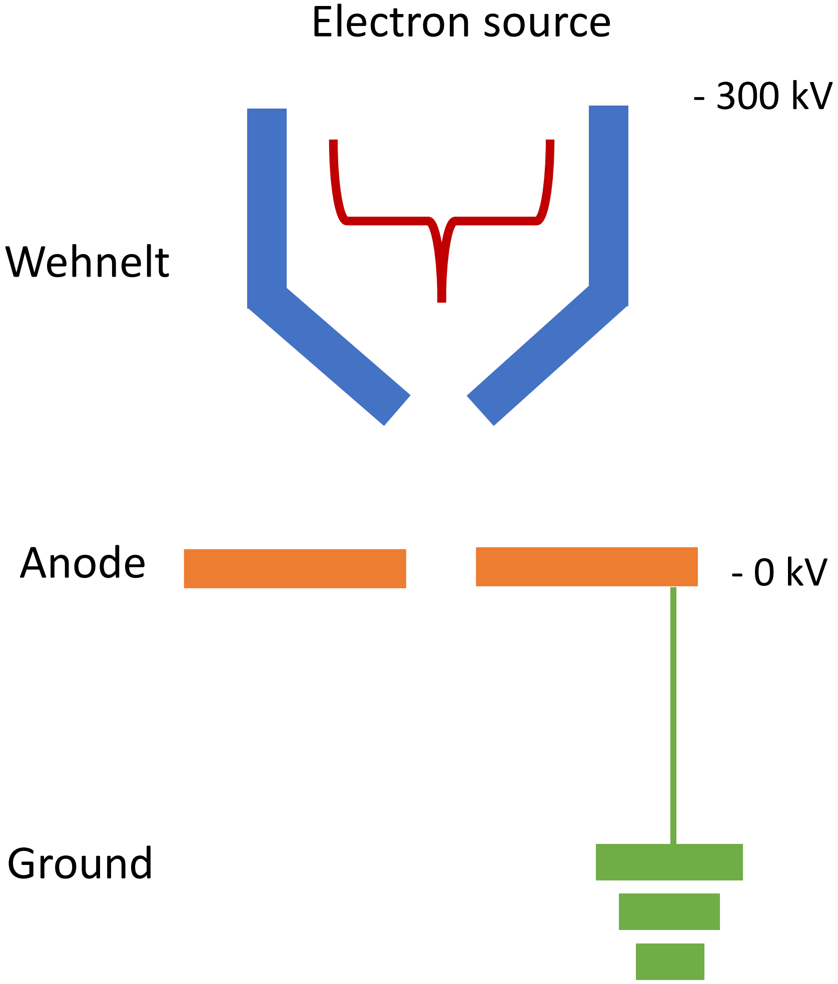


Example TEM schematic

One of many types of TEMs

myscope.training

Electron Gun



Electron source

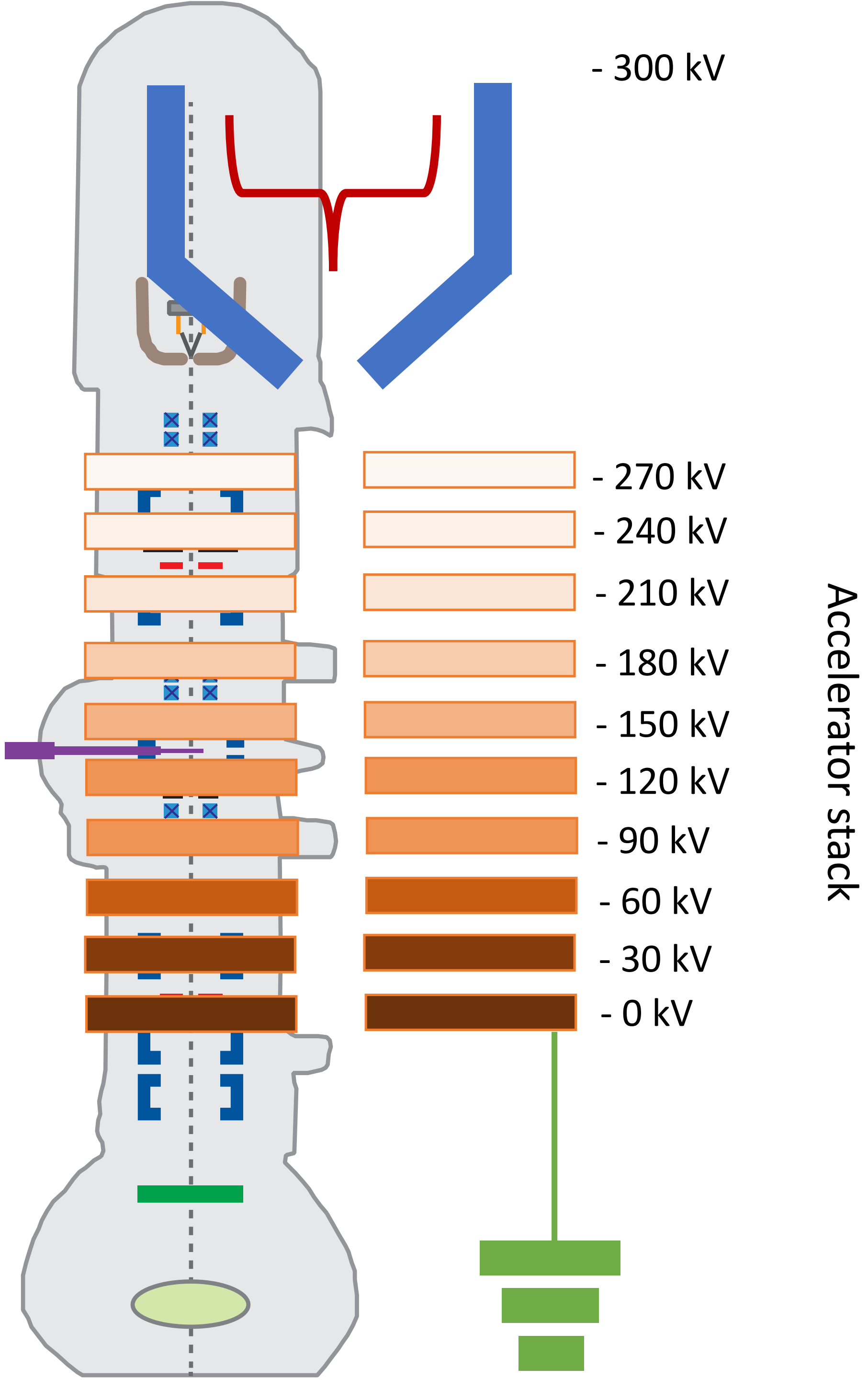
- 300 kV

Wehnelt

Anode

- 0 kV

Ground



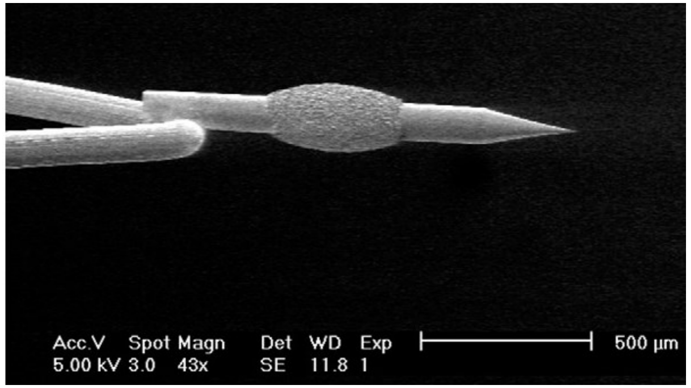
- 300 kV
- 270 kV
- 240 kV
- 210 kV
- 180 kV
- 150 kV
- 120 kV
- 90 kV
- 60 kV
- 30 kV
- 0 kV

Accelerator stack

Electron sources - types

- Thermionic
- Field emission

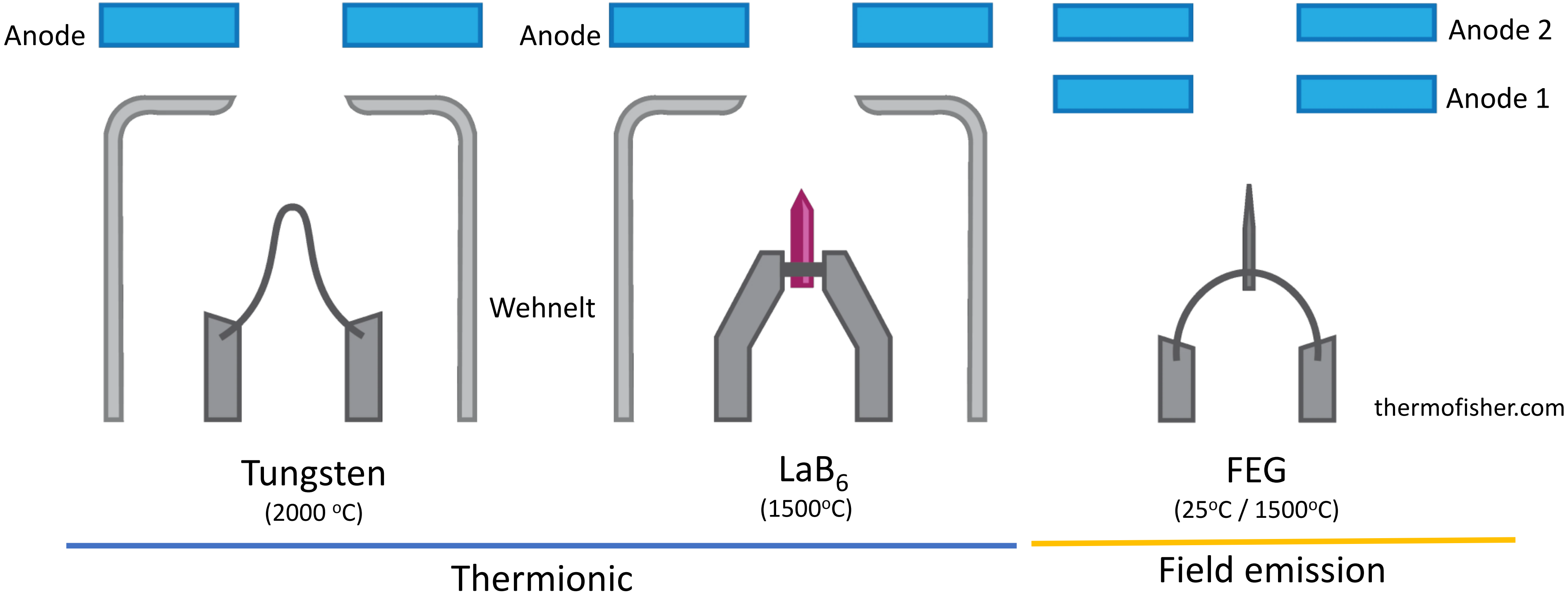
Schottky



Cook B.; Ultramicroscopy; 2009

Tungsten filament

microtonano.com



Anode

Anode

Anode 2

Anode 1

Wehnelt

Tungsten

(2000 °C)

LaB_6

(1500°C)

FEG

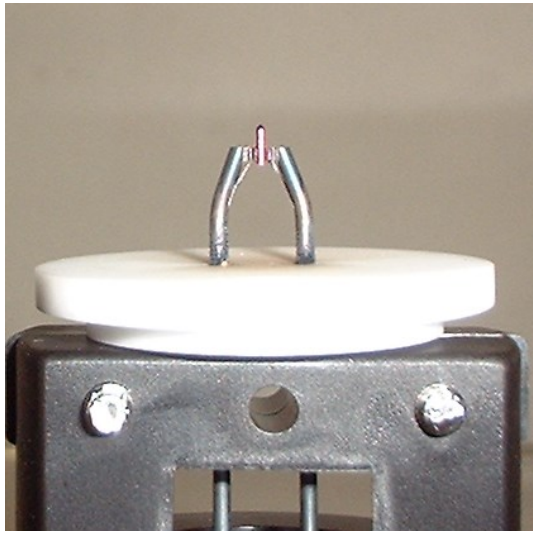
(25°C / 1500°C)

Thermionic

Field emission

thermofisher.com

LaB_6 crystall



snaggledworks.com

Electron sources - properties

Thermionic

- Tungsten filament
  - Low brightness
  - Inexpensive
- LaB_6 crystal
  - Higher brightness
  - Less energy spread
  - Lower lifetime

Field emission

- Schottky - warm
  - High brightness
  - Lower lifetime
  - Higher energy spread ☹
- Cold FEG
  - High brightness
  - Lower energy spread ☺
  - Lower stability

Electron Gun Properties

Source	**Brightness**	**Stability**(%)	**Size**	**Energy spread**	**Vacuum**
W	$3X10^5$	~1	50μm	3.0(eV)	10^{-5} (τ)
LaB_6	$3x10^6$	~2	5μm	1.5	10^{-6}
C-FEG	10^9	~5	5nm	0.3	10^{-10}
T-FEG	10^9	<1	20nm	0.7	10^{-9}

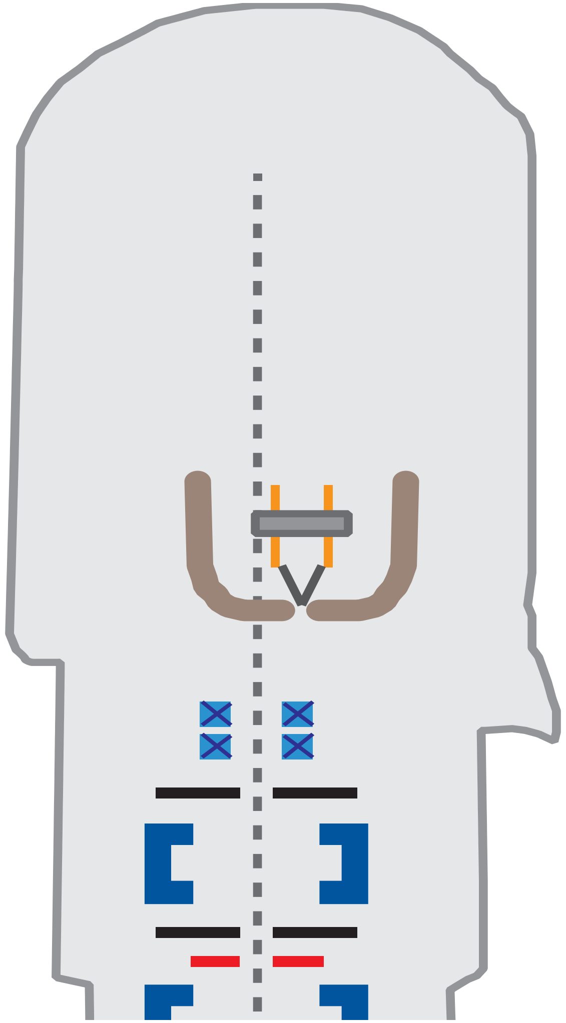
Brightness – beam current density per unit solid angle

mse.iastate.edu

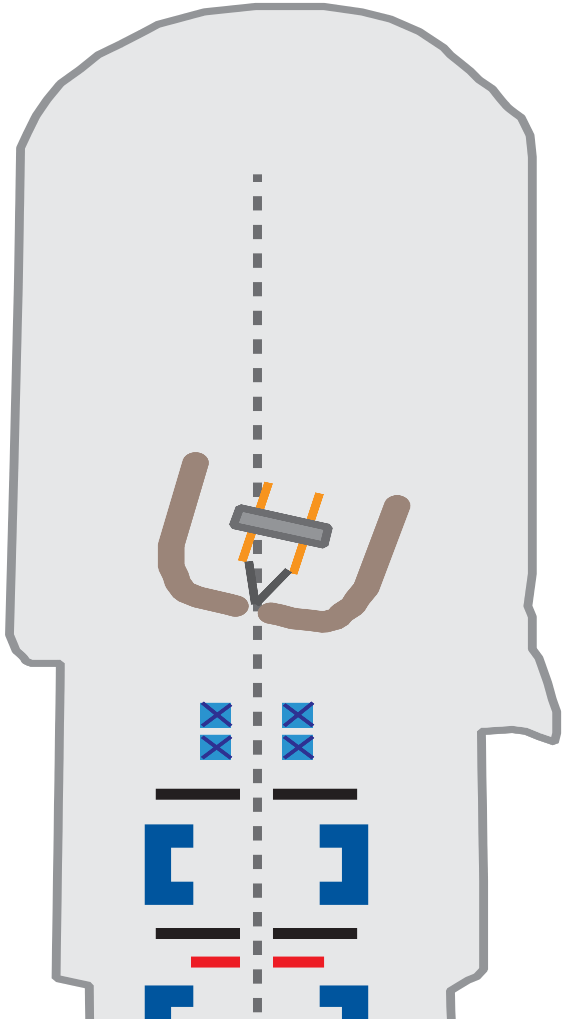
Electron beam properties

- Beam coherence
- Wave-length: at 300 kV => ~2 pm = 0.002 nm = 0.02 Å
- Velocity: at 300 kV => 0.76c
- Magnetic momentum
- Can be focused
- Disadvantage: hi interaction rate (small penetration)

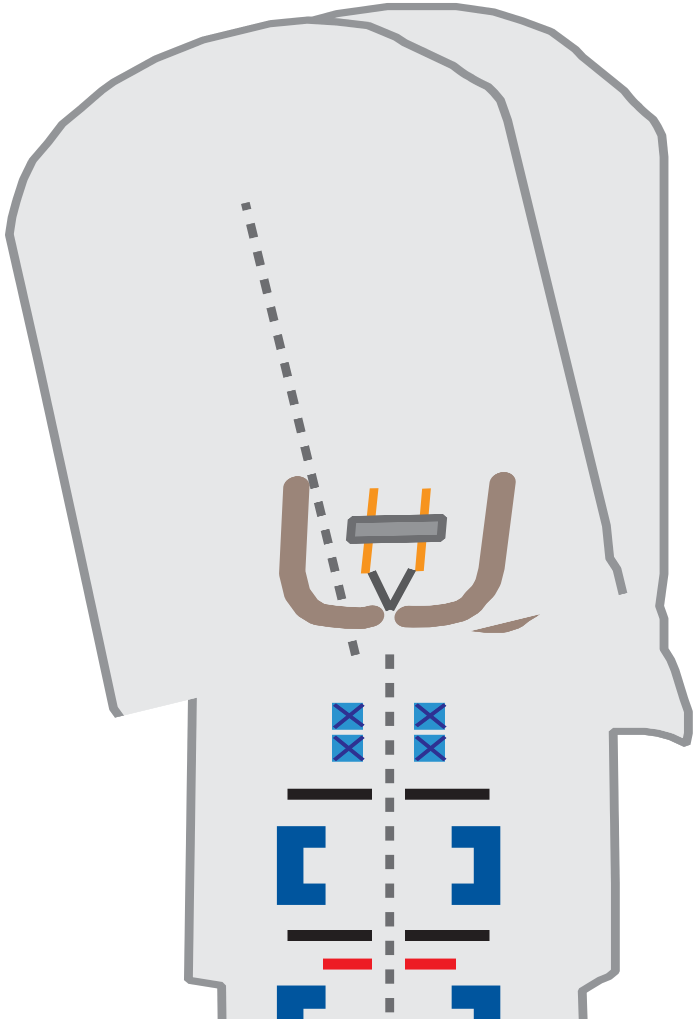
Electron beam deflectors



Gun is shifted



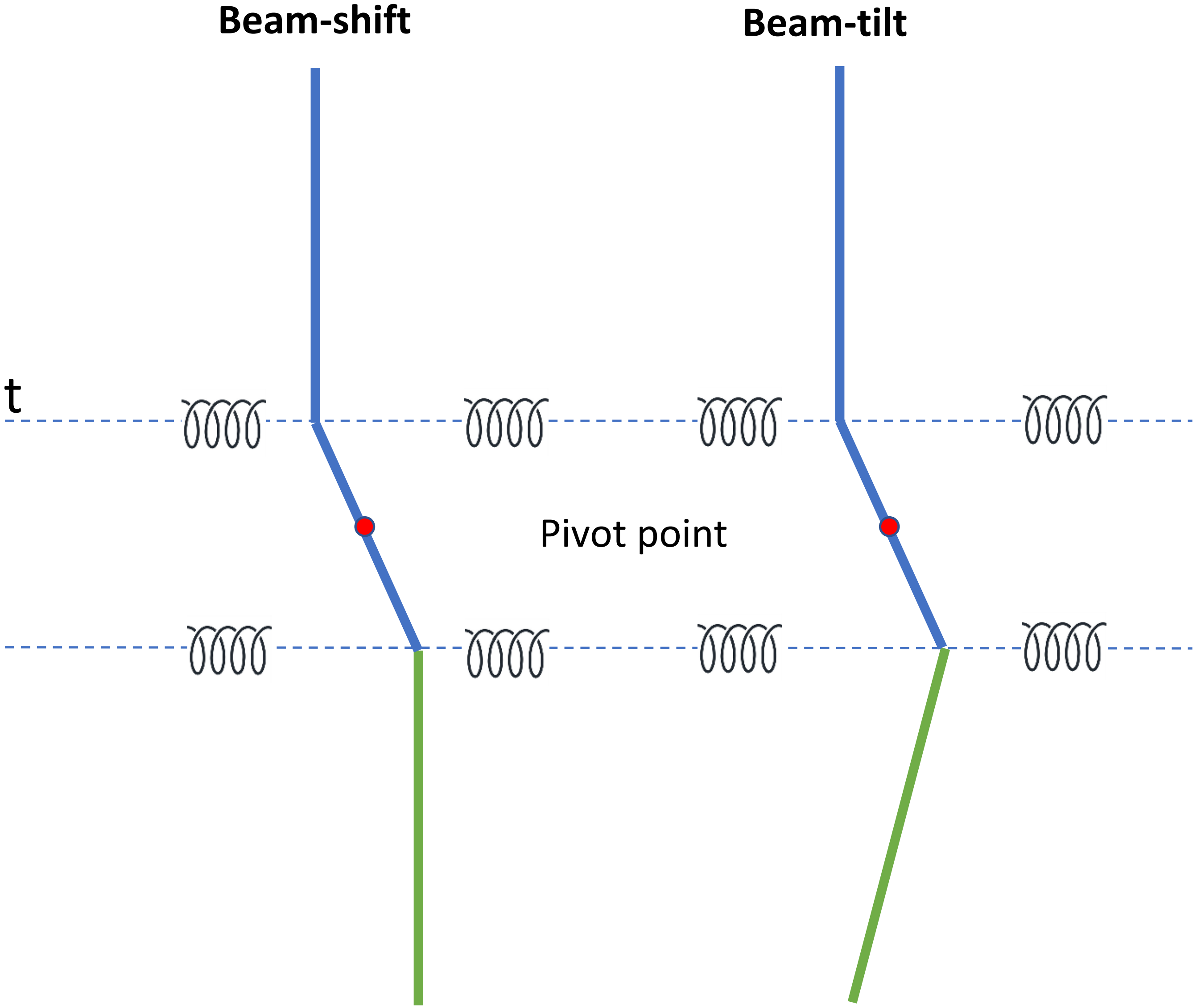
Gun is tilted



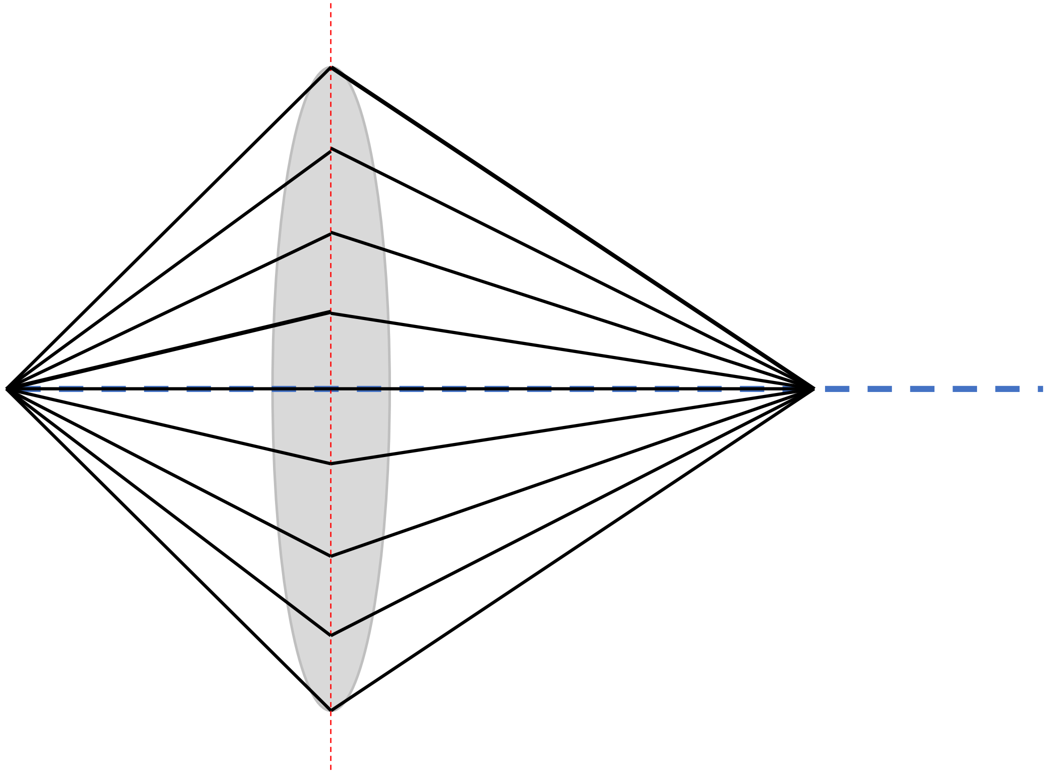
Gun is shifted and tilted

Electron beam deflectors

- Electron has a magnetic momentum
- We can move electrons by magnetic field
- We can generate magnetic fields by electric coils
- The same coils controls the shift and tilt
- Movement of the beam in 2D (X,Y)
- Tuning the pivot points:
  - Tilt free shift
  - Shift free tilt

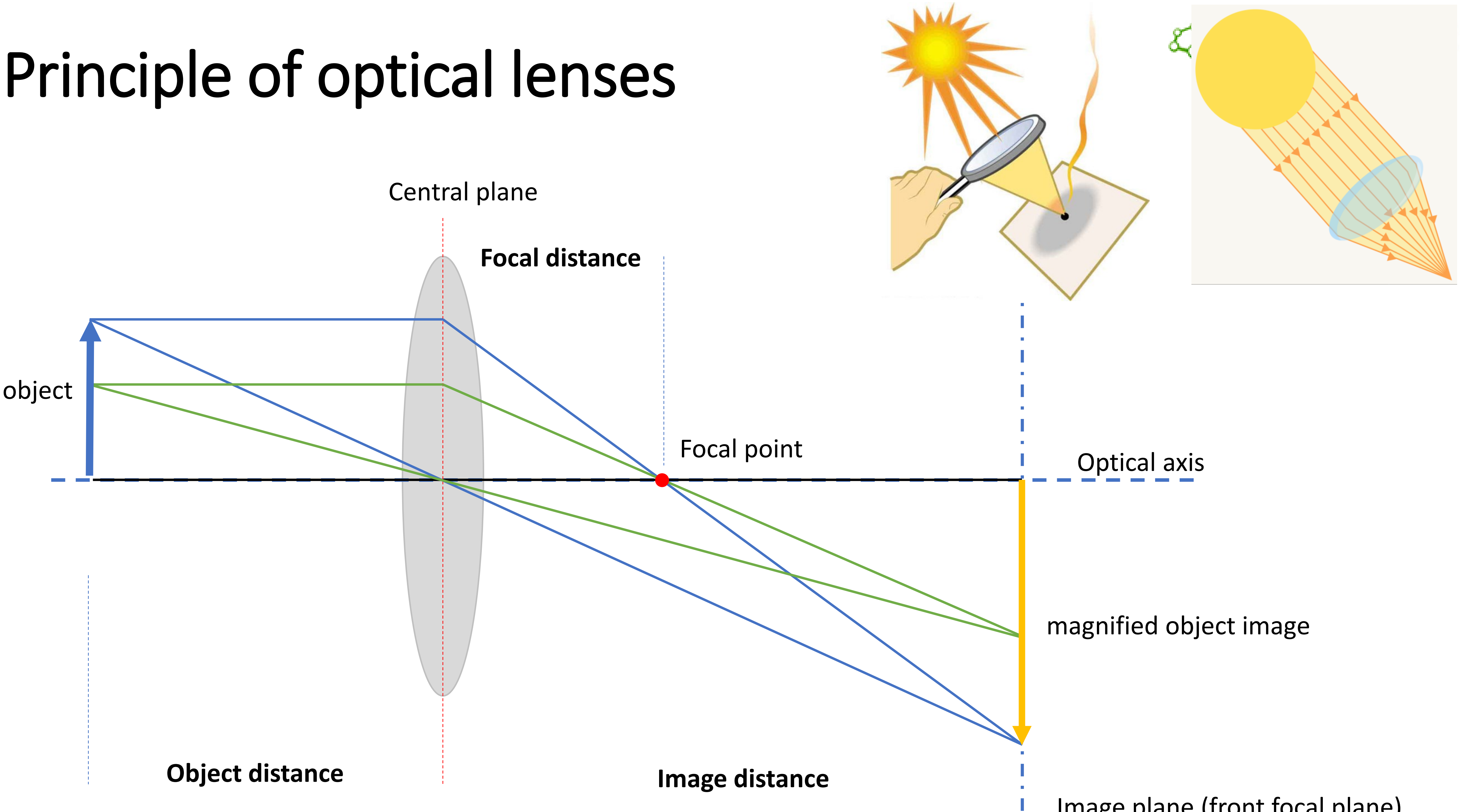


Principle of convex optical lenses



- Ray passing through optical axis remains unbent
- Rays passing through lens are bent that they focus (converge) in focal point

Principle of optical lenses



Central plane

Focal distance

object

Focal point

Optical axis

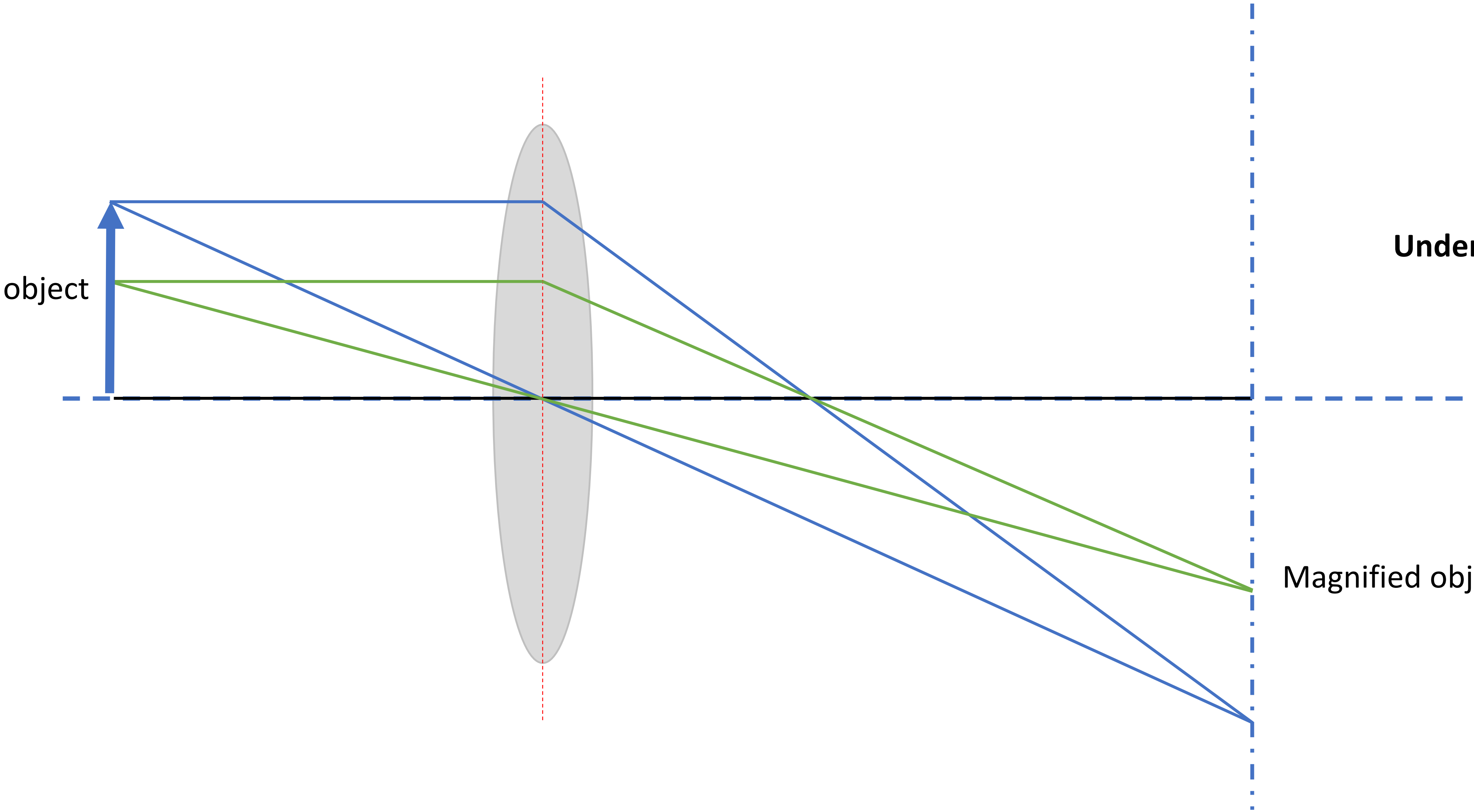
magnified object image

Object distance

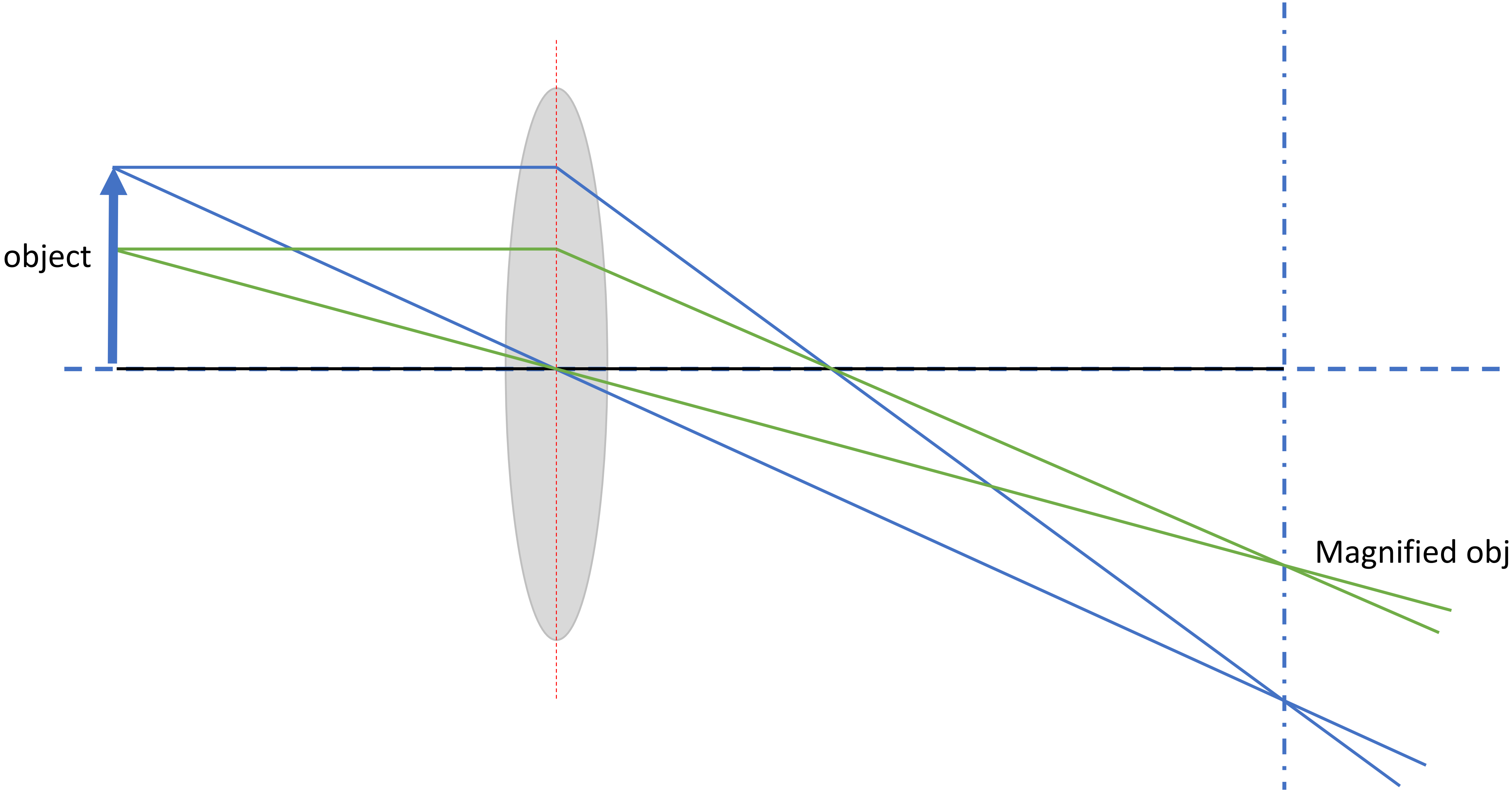
Image distance

Image plane (front focal plane)

Principle of optical lenses

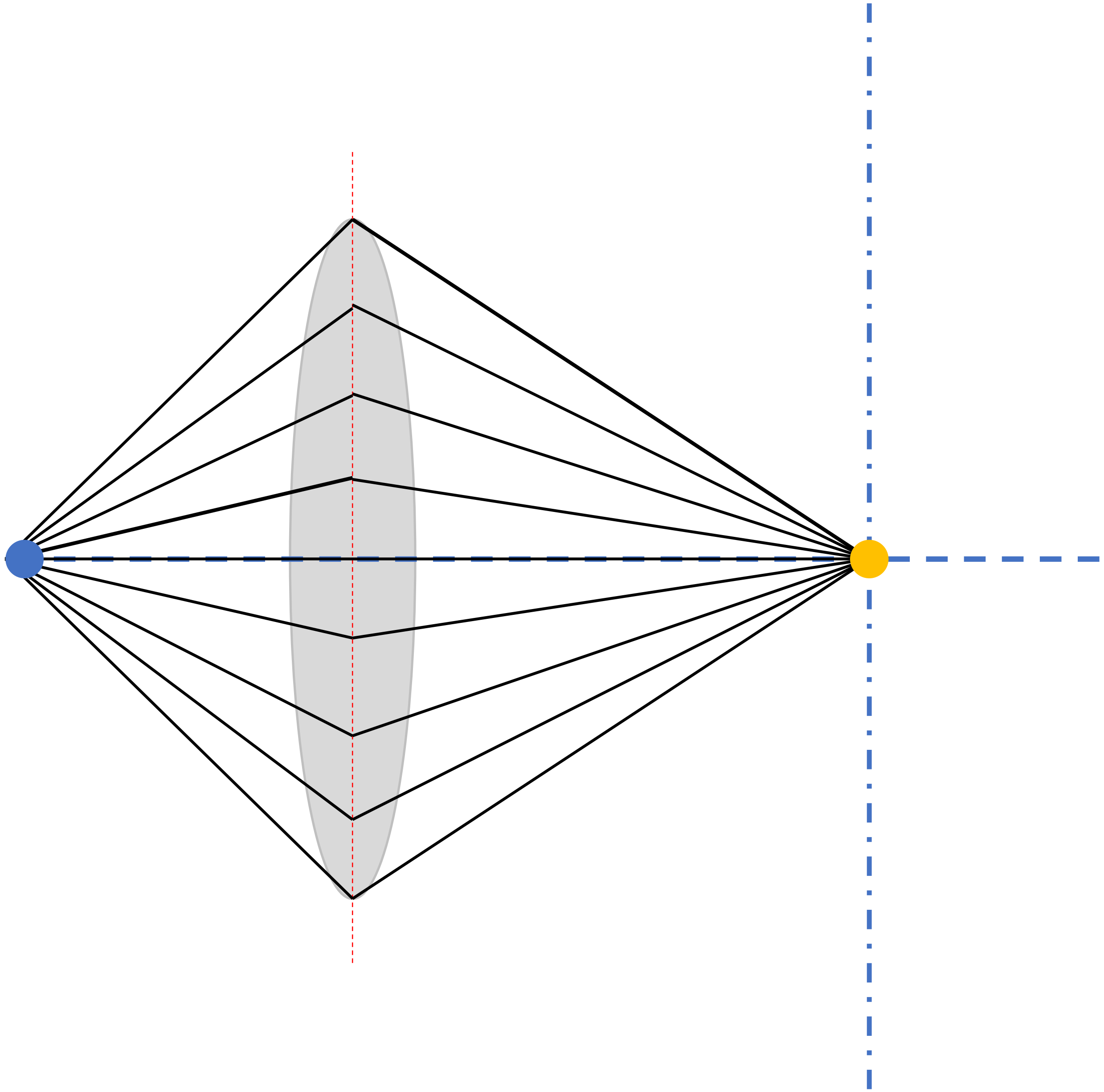


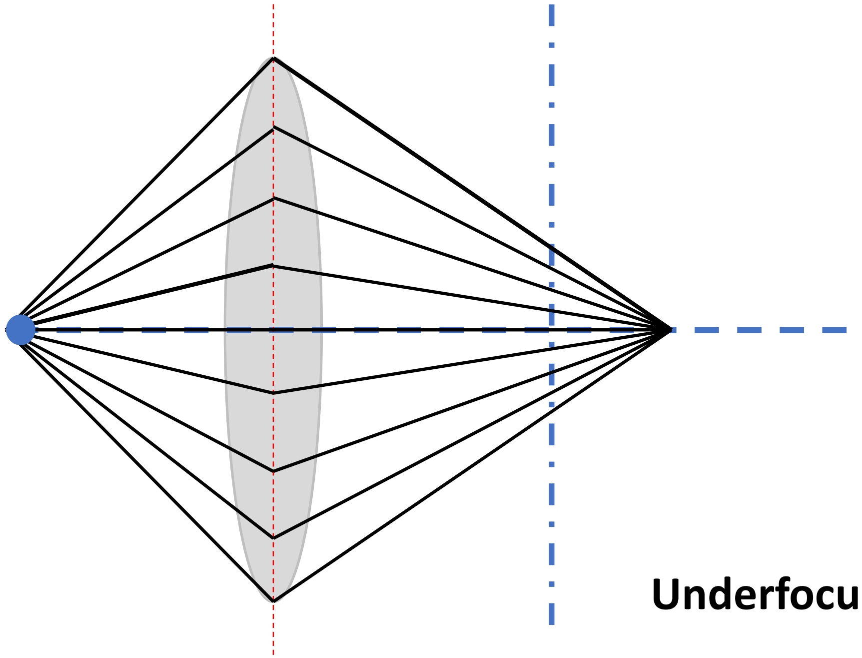
Principle of optical lenses



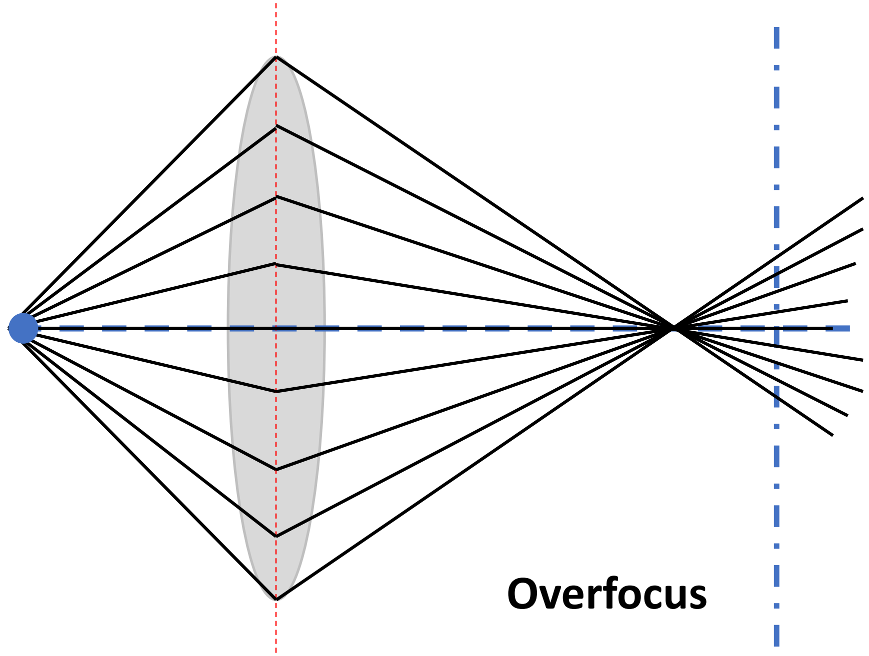
Overfocus

Alternative way of showing lenses





Underfocus

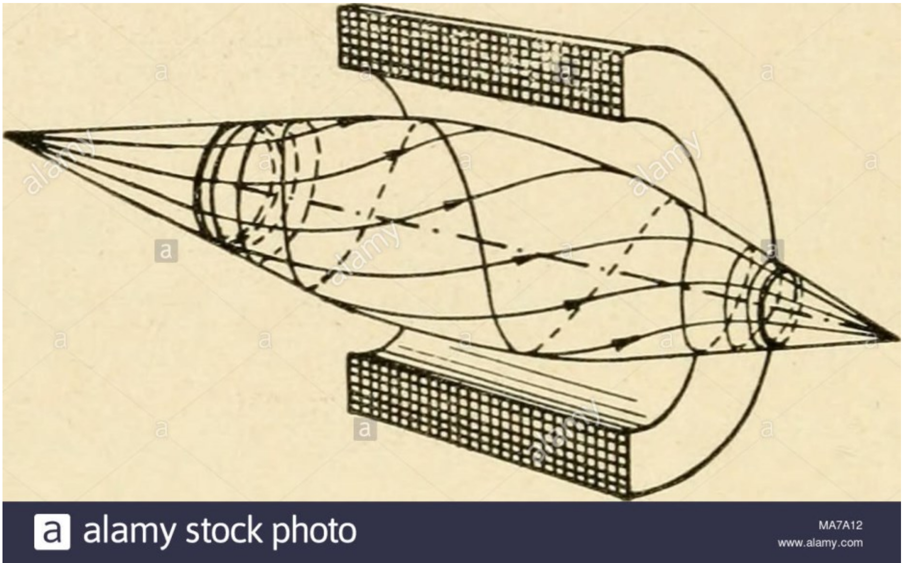


Overfocus

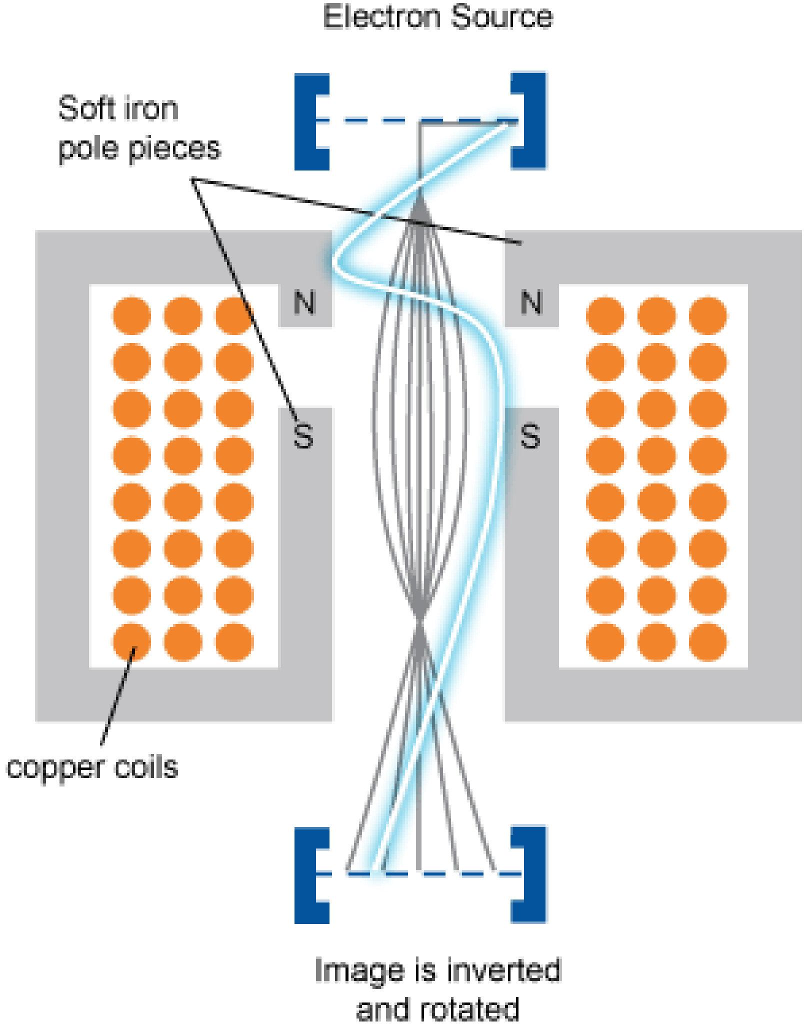
How electromagnetic lenses work

Lorentz force

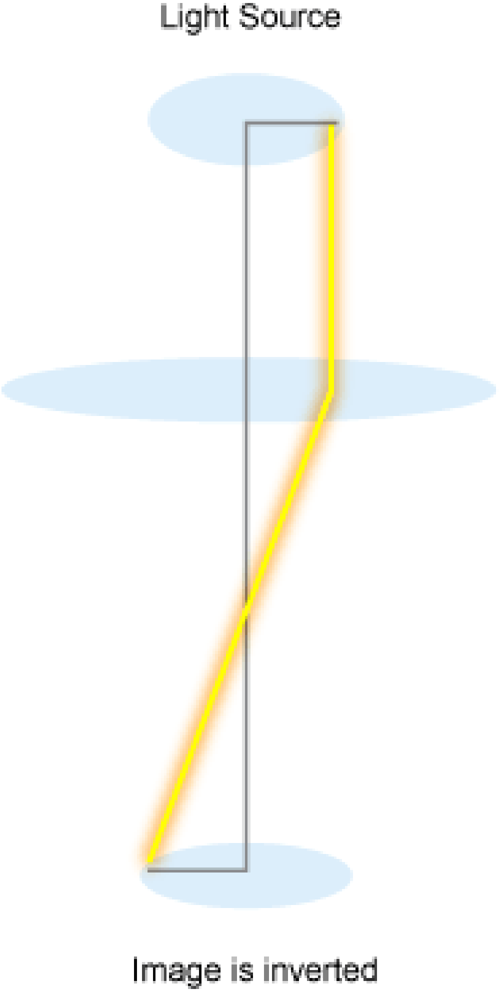
$$\mathbf{F} = q\,\mathbf{E} + q\,\mathbf{v} \times \mathbf{B}$$



Magnetic lens



Optical lens



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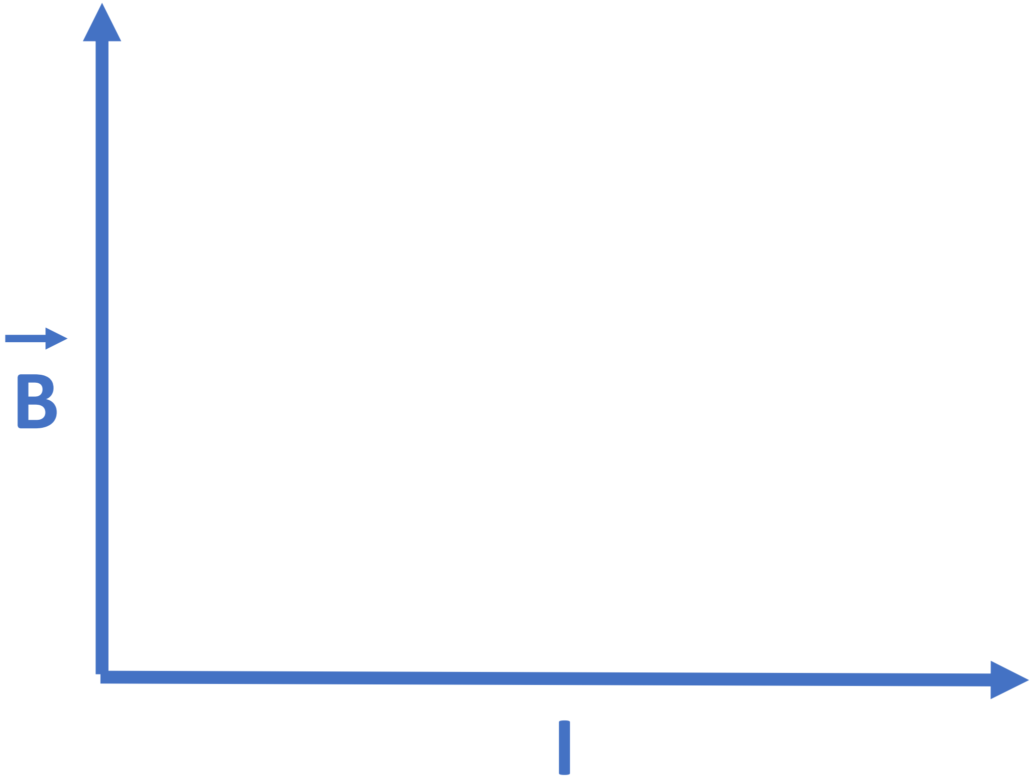
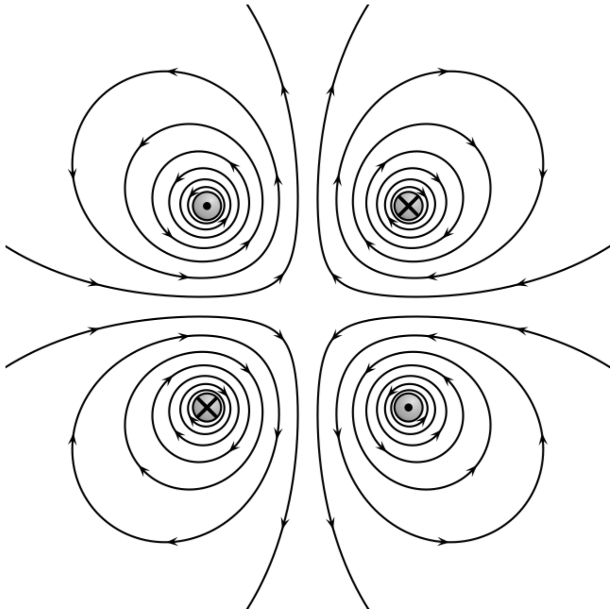
Electromagnetic lenses - properties

- Variable strength
- Hysteresis
- Non-homogenity of the coil current => magnetic field => aberrations

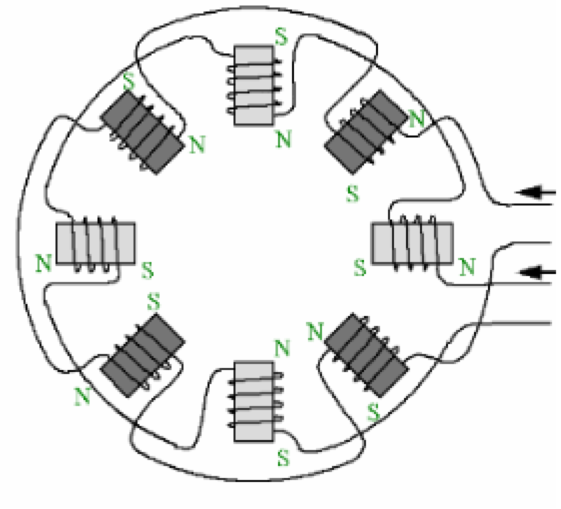


How to correct for aberrations

- Astigmatism
  - Defocus in one direction is different then in the other
  - Stigmator
    - Quadrupole magnetic field
    - Octapole stigmator (still quadrupole magnetic field)
- Higher order aberrations
  - Non-homogeneity of the magnetic field
  - High frequencies affected more
  - Cs corrector

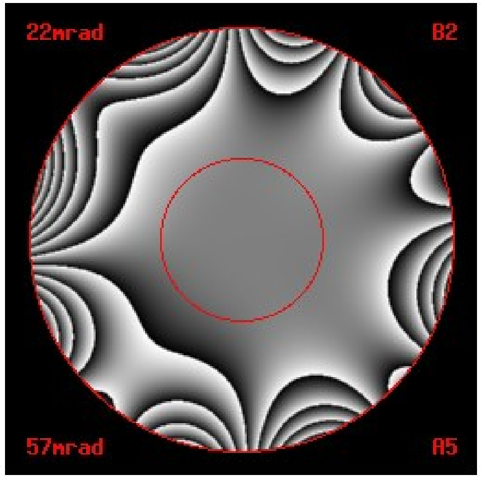


wikiwand.com



jeol.jp

Ceos-gmbh.de



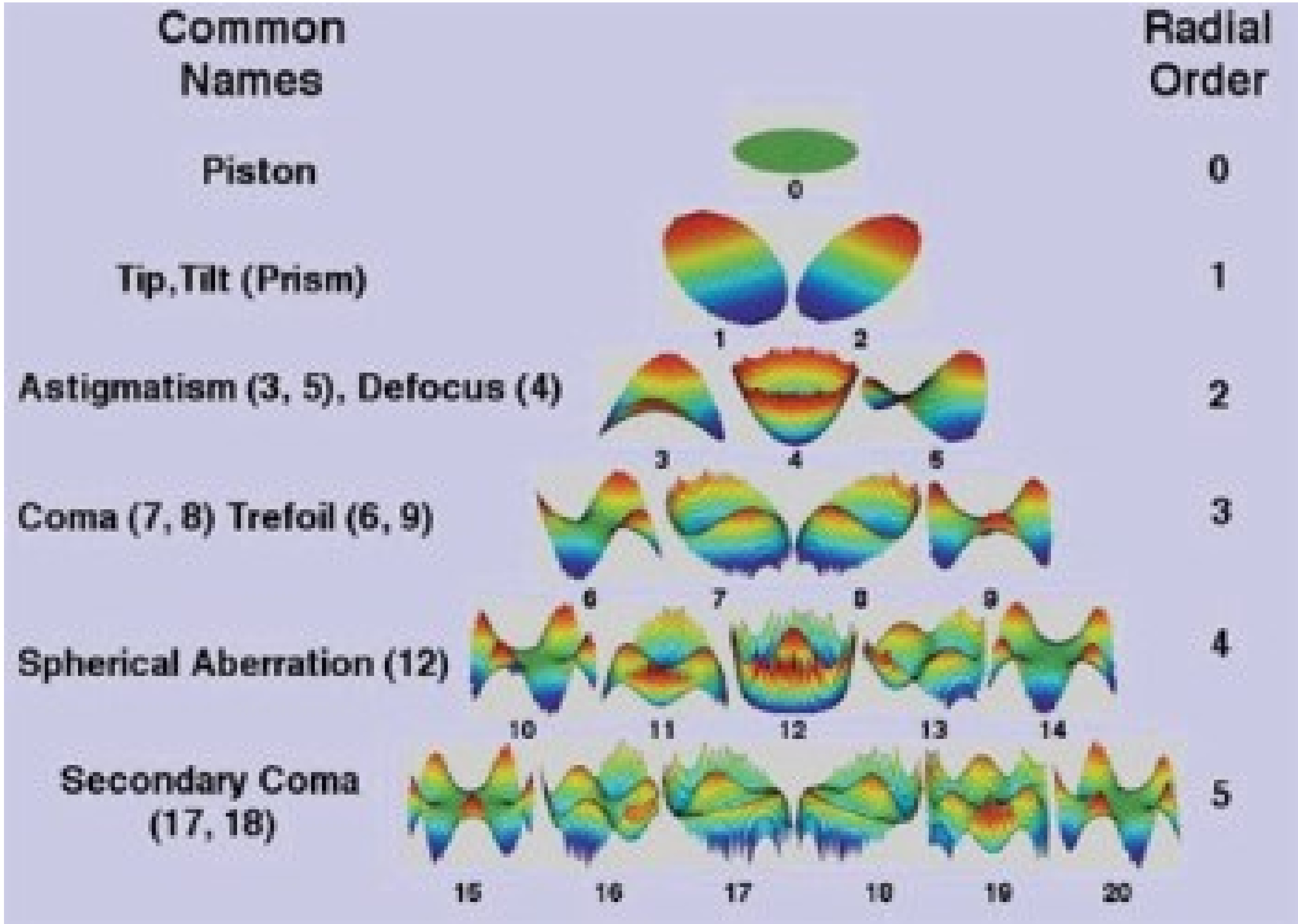
Ceos-gmbh.de

Mathematatic description of aberrations

Zernike polynomials

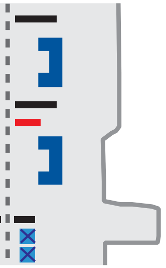
Frits Zernike

Nobel Prize 1953 (phase contrast microscopy)

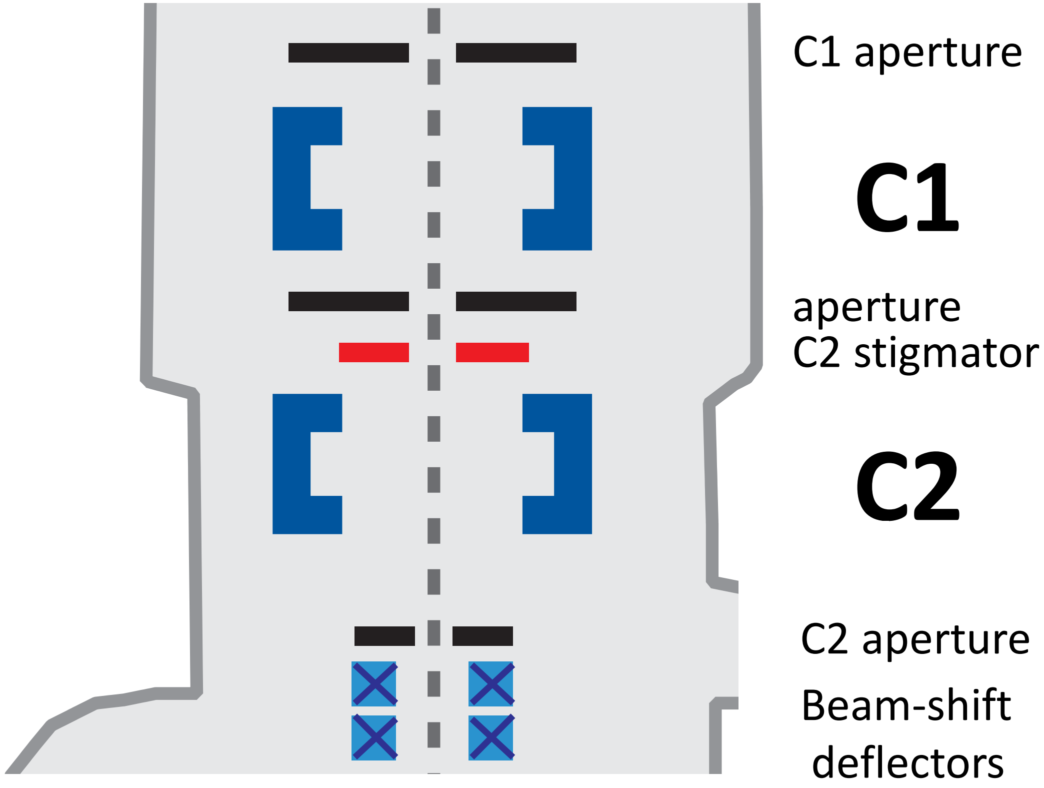


your-eye-sight.org

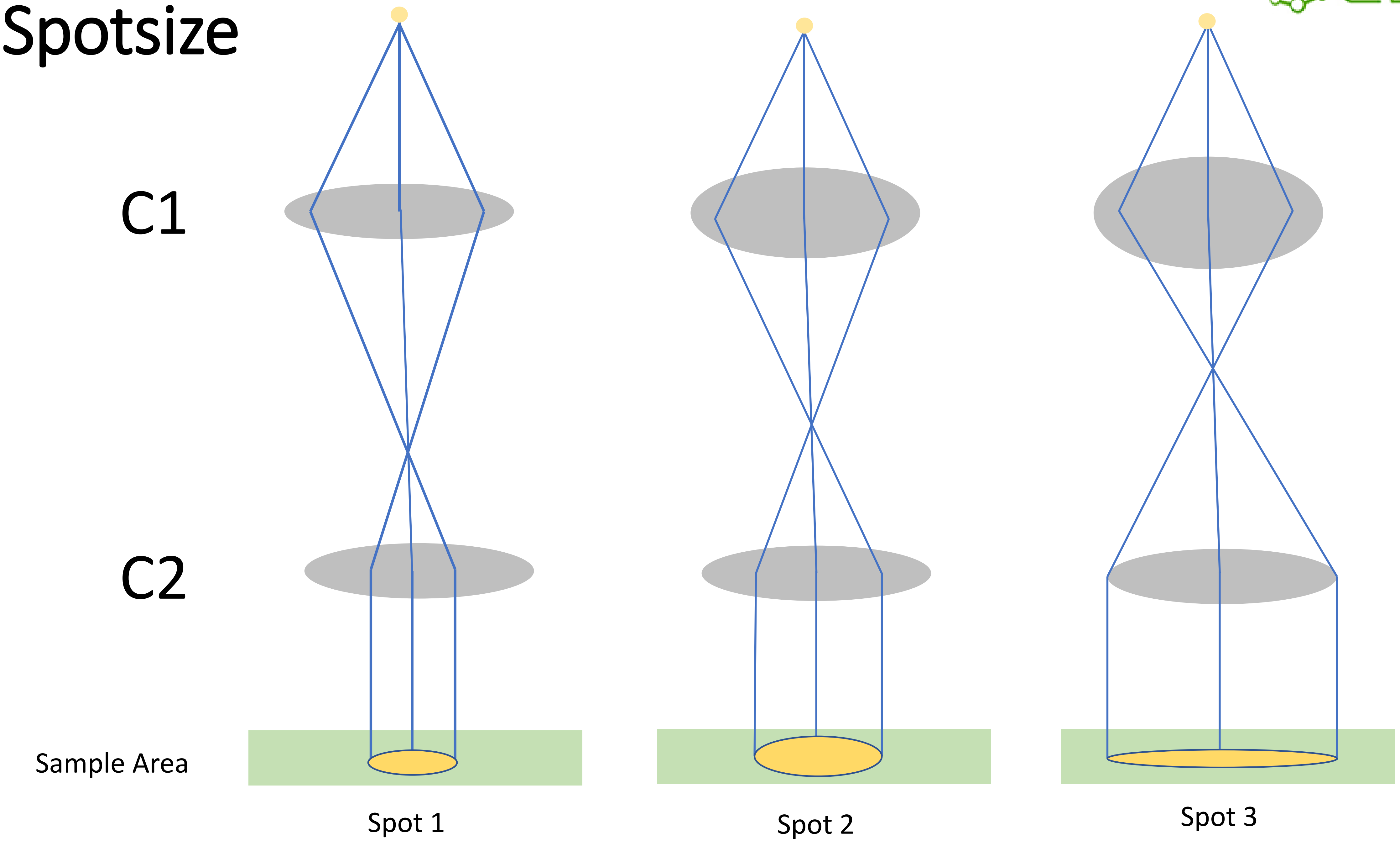
Illumination system (Condenser system)



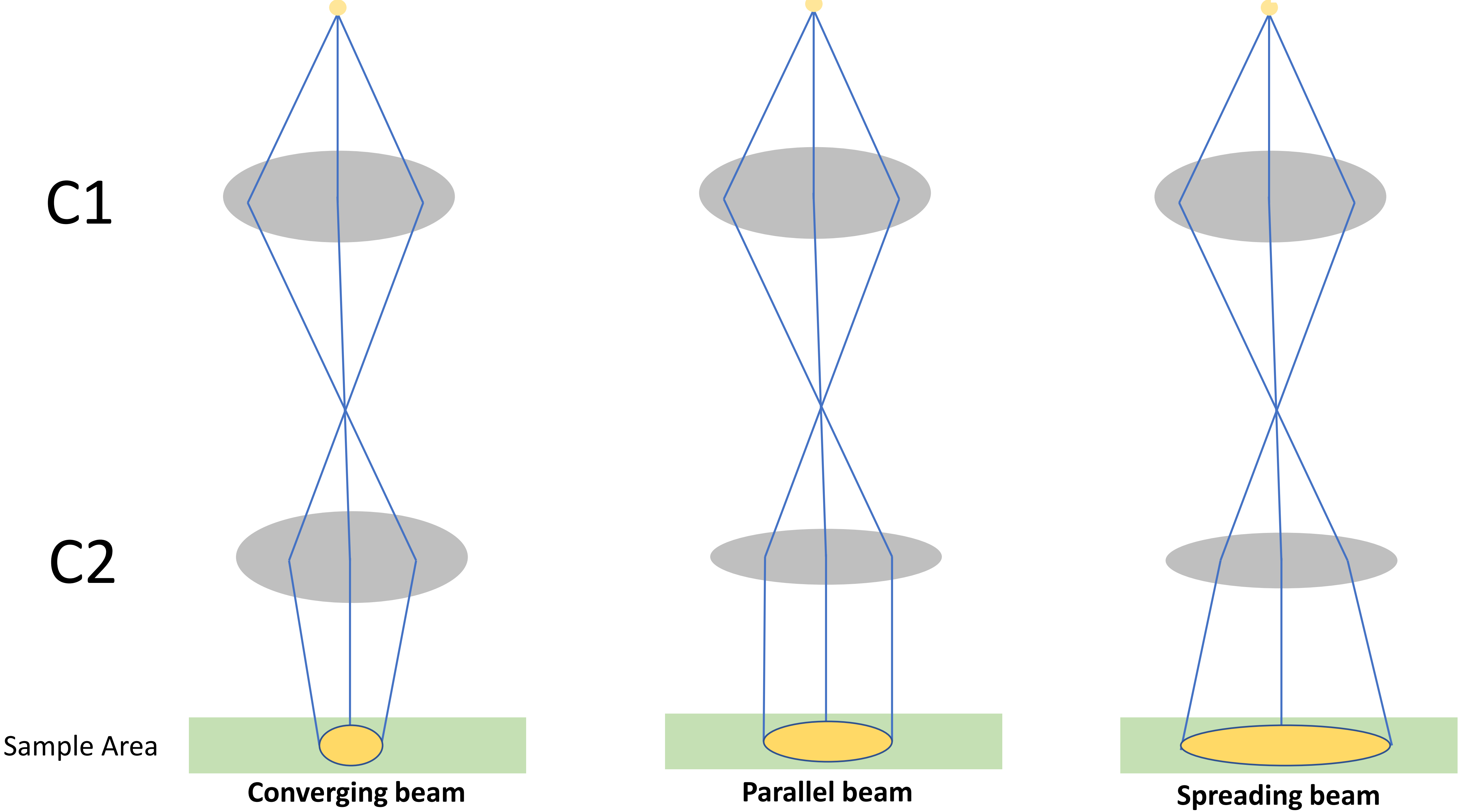
- Condenser lenses
  - 2 condenser system
    - C1 lens – spot size
    - C2 lens – beam size (diameter)
  - 3 condenser system
    - C2/C3 – beam size (diameter)
- Condenser apertures
- Beam-shift
  - Deflectors
- Goal for hi-res microscopy
  - Parallel illumination beam
  - Controlled dose/flux



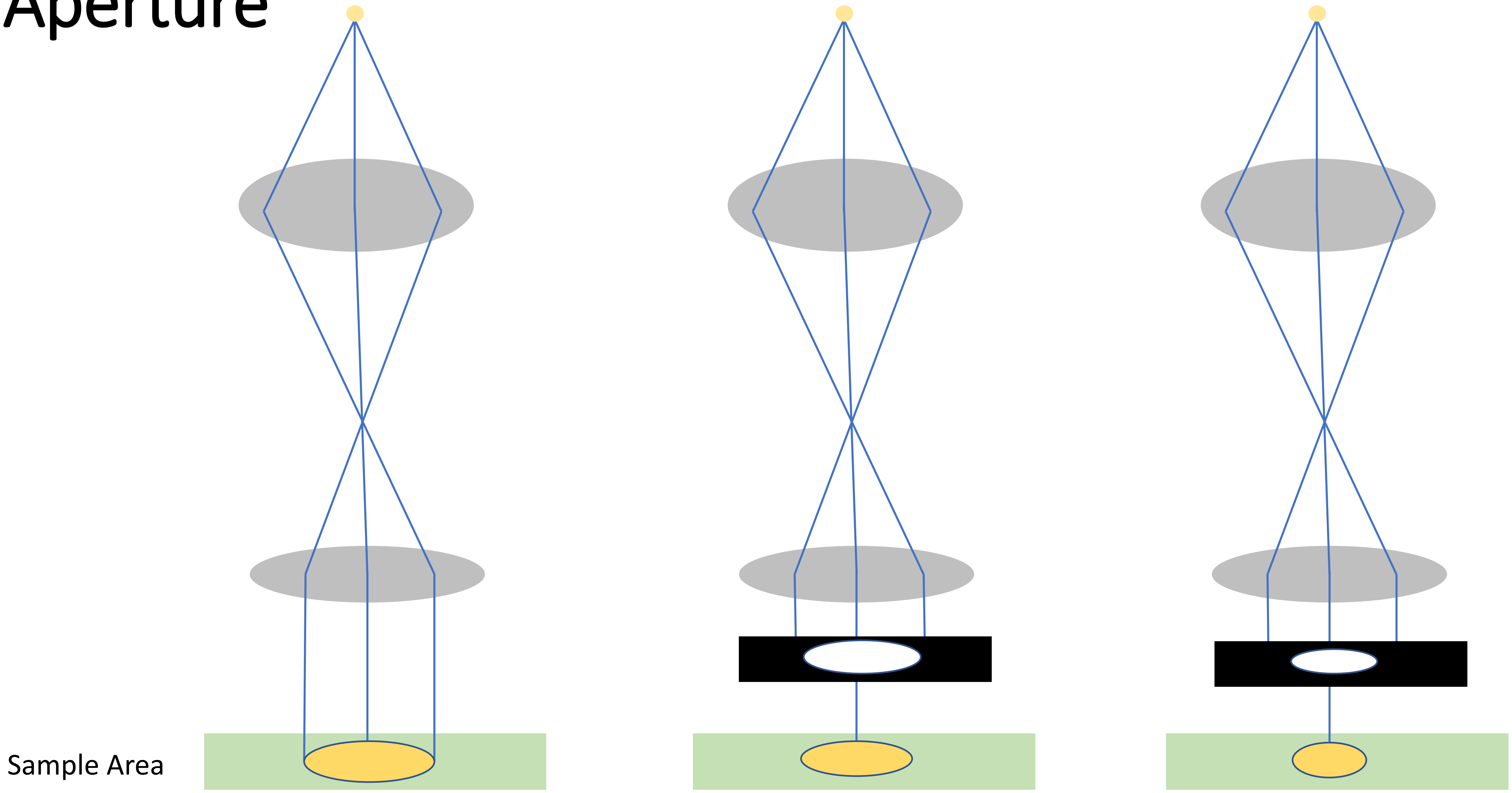
Spotsize



Intensity


C1
C2
Sample Area
Converging beam
Parallel beam
Spreading beam

Aperture

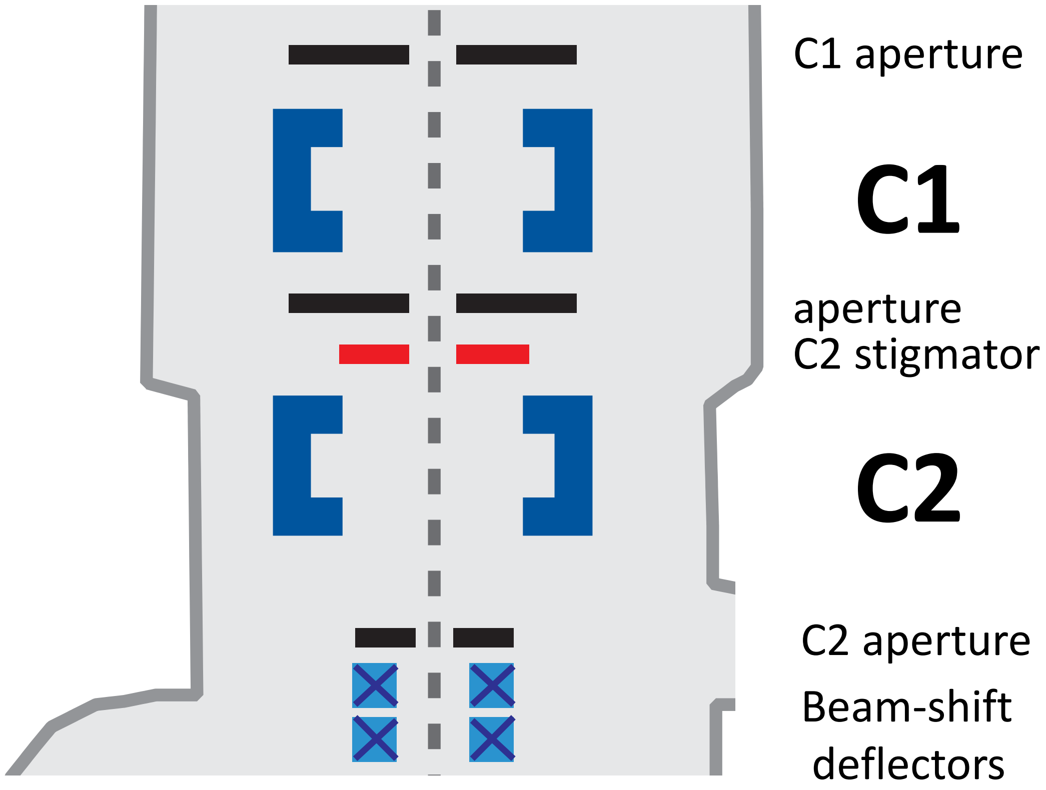


No apperture

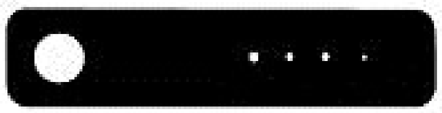
“Big” apperture

“Small” apperture

C1 lens, C2 lens, C2 aperture

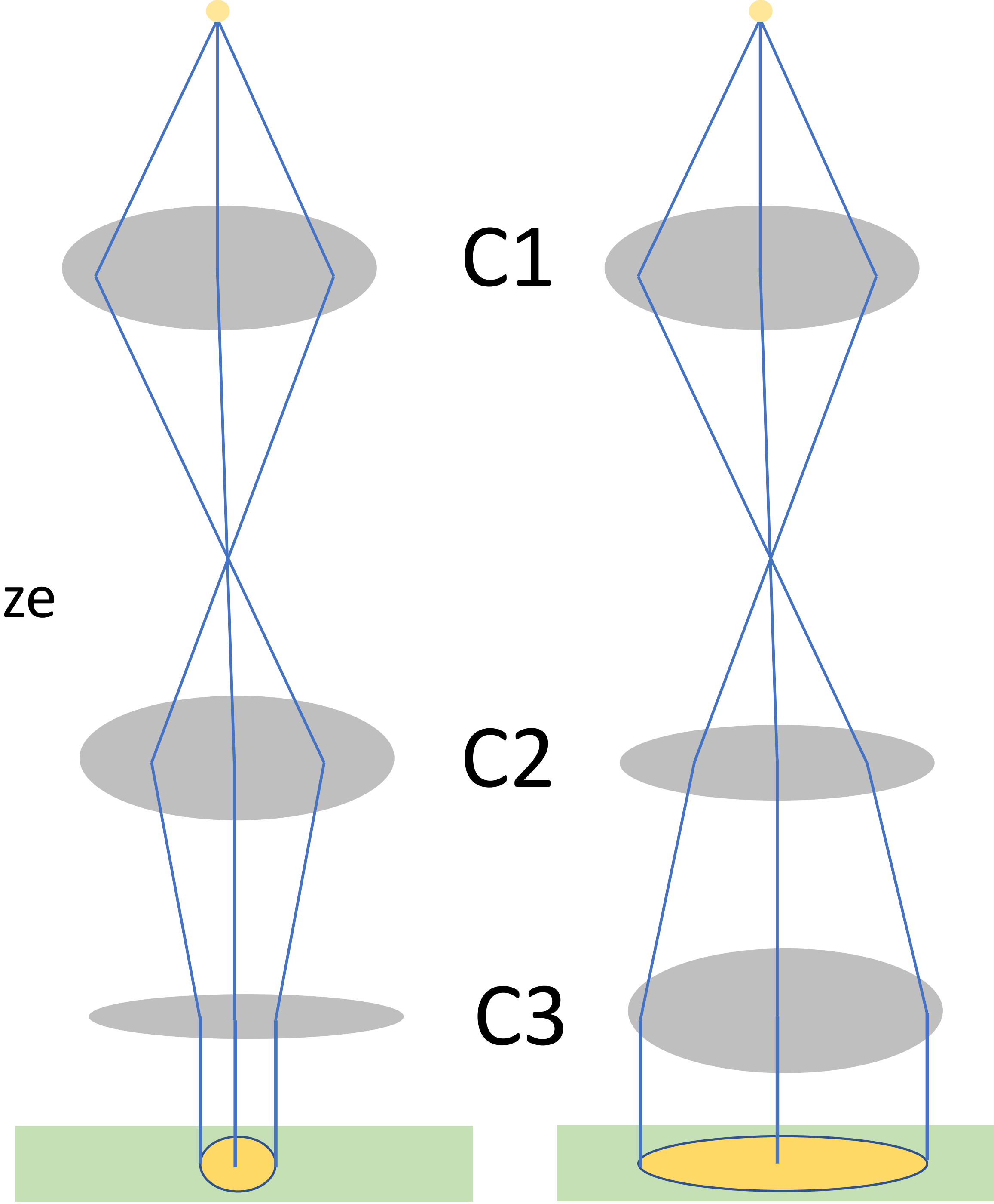


- C1 lens
  - Strength changed in steps
  - Controlled by “Spotsize”
  - Major impact on electron flux
- C2 lens
  - Strength change gradually
  - Controlled by “Intensity”, “Brightness”
  - Controls the convergence of the beam
  - Converging beam higher electron flux than diverging
- Aperture
  - Cuts out part of the beam after C2
  - Reduces illuminated area but not the flux



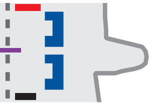
A three-condenser system

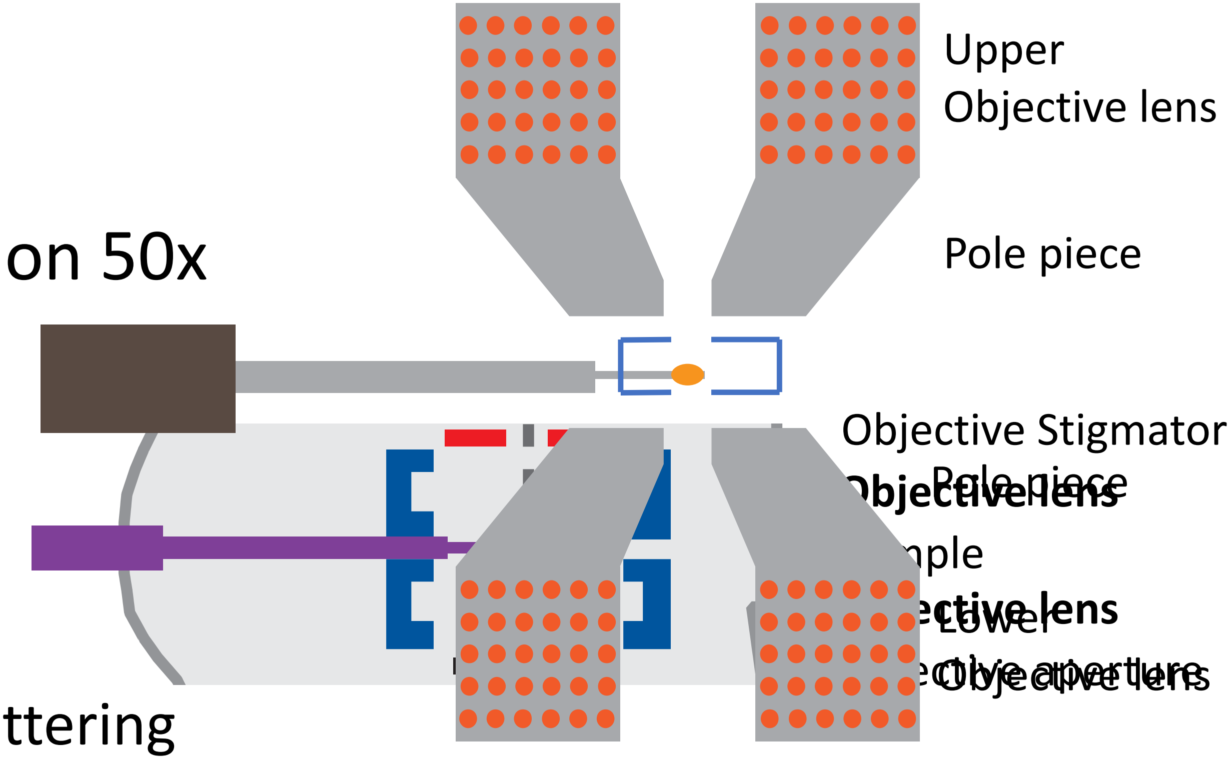
- Hi-end microscopes
- Behind C2 aperture
- Parallel beam across wide range of beam size
- Precise setting of dose and beam size
- Easier setup for phase plate usage
- Calibrate the ratio between C2 and C3



Objective lens system

- Objective lens – highest magnification 50x
  - Strength set by “Focus”
- Objective stigmator
  - Remove two-fold astigmatism
- Objective aperture
  - Remove electrons with high angle scattering angle
  - Improves contrast
  - Has influence on resolution (cut-off)
  - Has influence on astigmatism



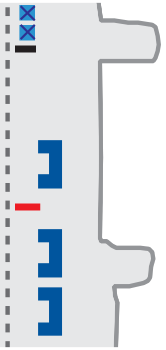


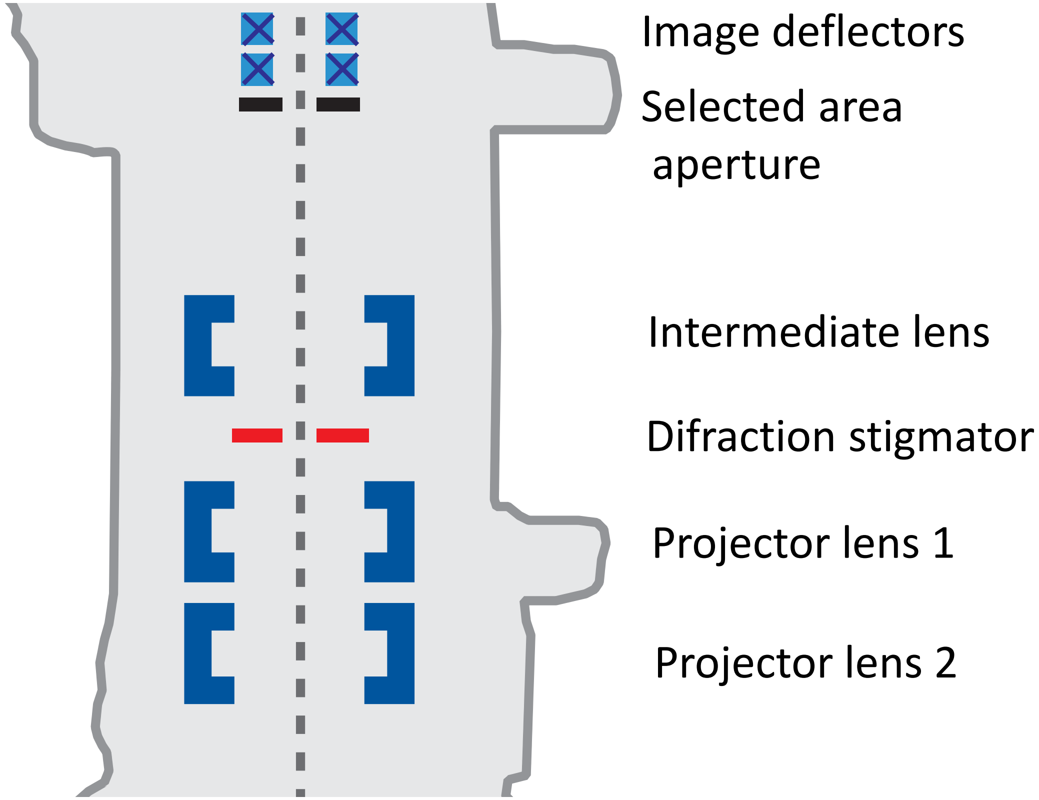
Sample stage

- Moving in 3 dimensions (X, Y, Z)
  - Eucentric height
- Rotation (tilt)
  - Sample centered
  - goniometer
- Cooled at liquid nitrogen temp
- Stability crucial for hi-res
- Cryo-decontamination box

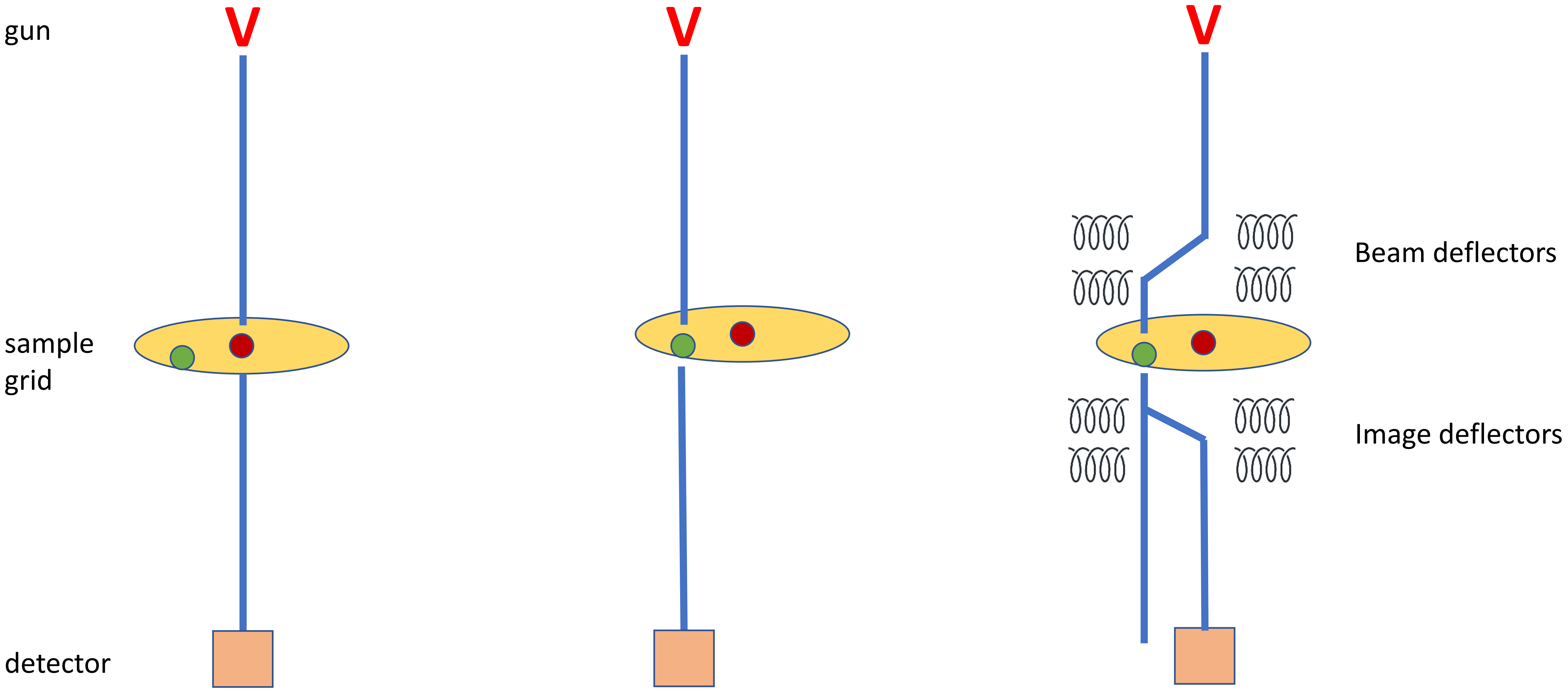
Projector system

- Intermediate lens and Projector lens
  - Intermediate magnification 10-20x
- Final magnification of the image
- Rotation free lenses
- Magnification modes
  - LowMag
  - SA
  - EFTEM
- Image deflectors
  - Shifts the beam-shifted image back to detector





Stage-shift vs image-shift (beam-shift)



gun

sample grid

detector

Beam deflectors

Image deflectors

Original position

Stage-shift

Image-shift

Stage-shift vs beam-shift/image-shift

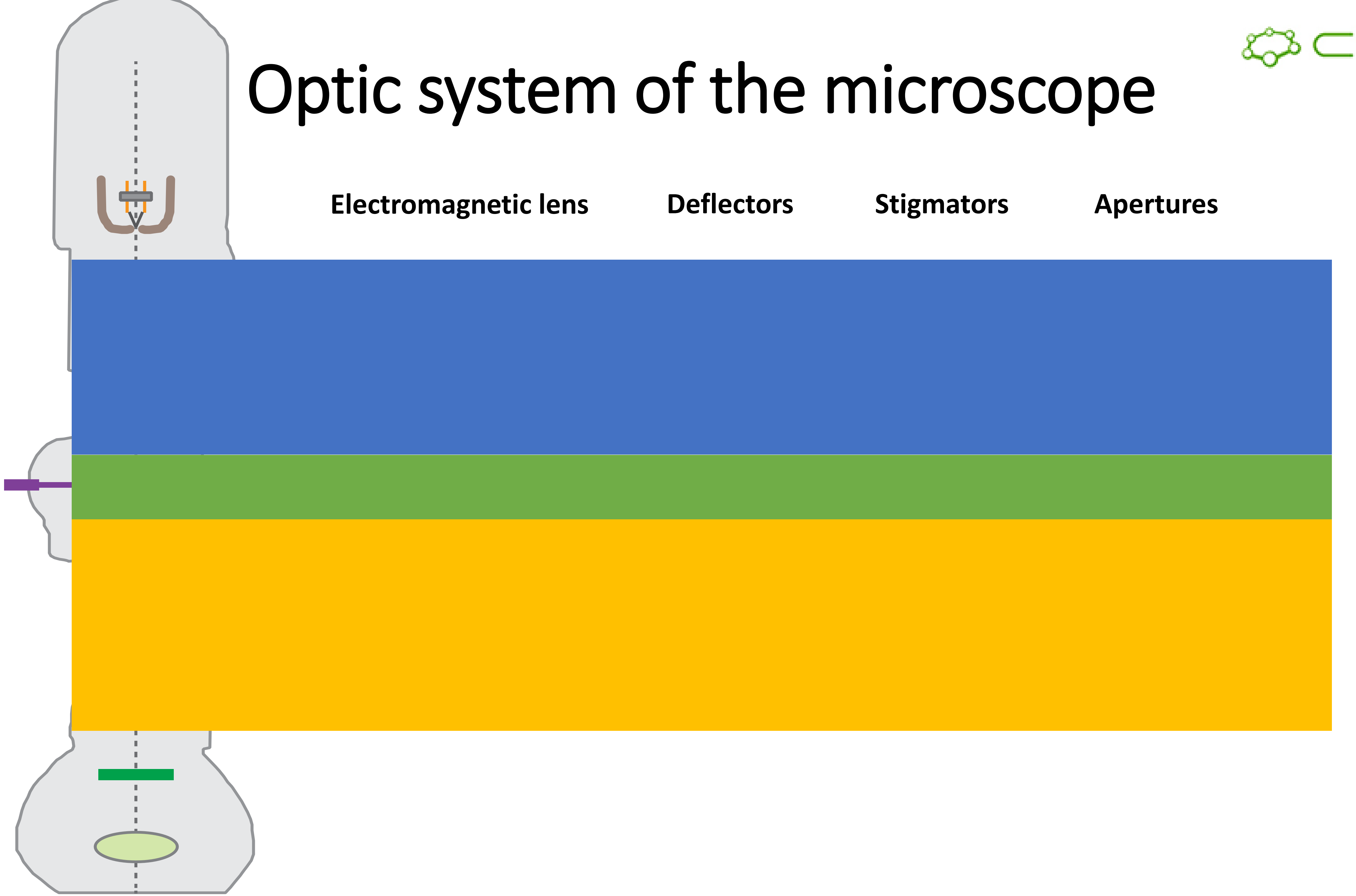
Stage-shift

- Mechanical
- Takes time to stabilize (~15 s)
- Large movements (+- grid surface)
- Small precision

Beam-shift/Image-shift

- Electro-magnetic
- Faster stabilization (~5 s)
- Small movements (~5 µm)
- High precision
- Introduces beam-tilt

Optic system of the microscope

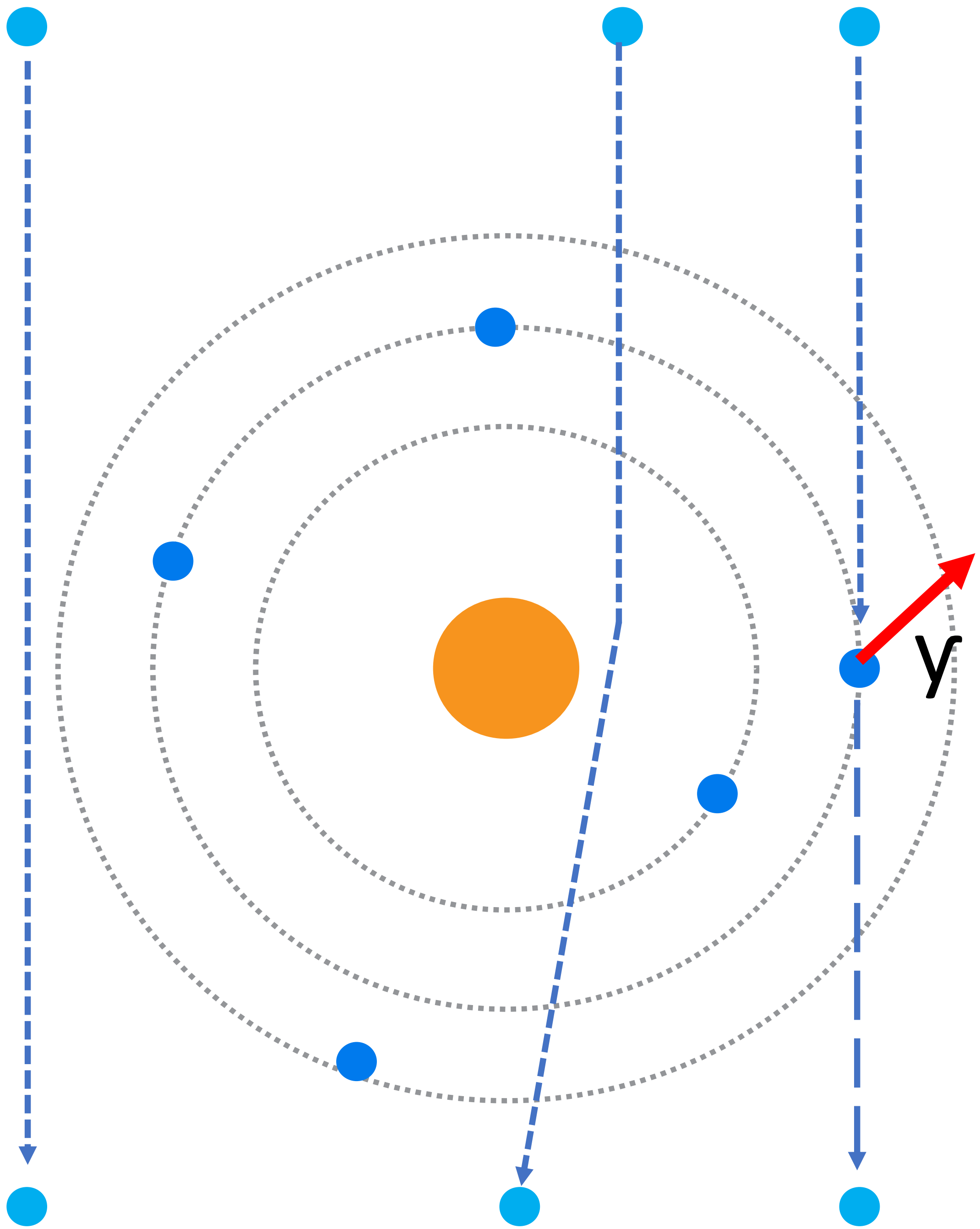


	Electromagnetic lens	Deflectors	Stigmators	Apertures
Condenser				
Objective				
Projector				

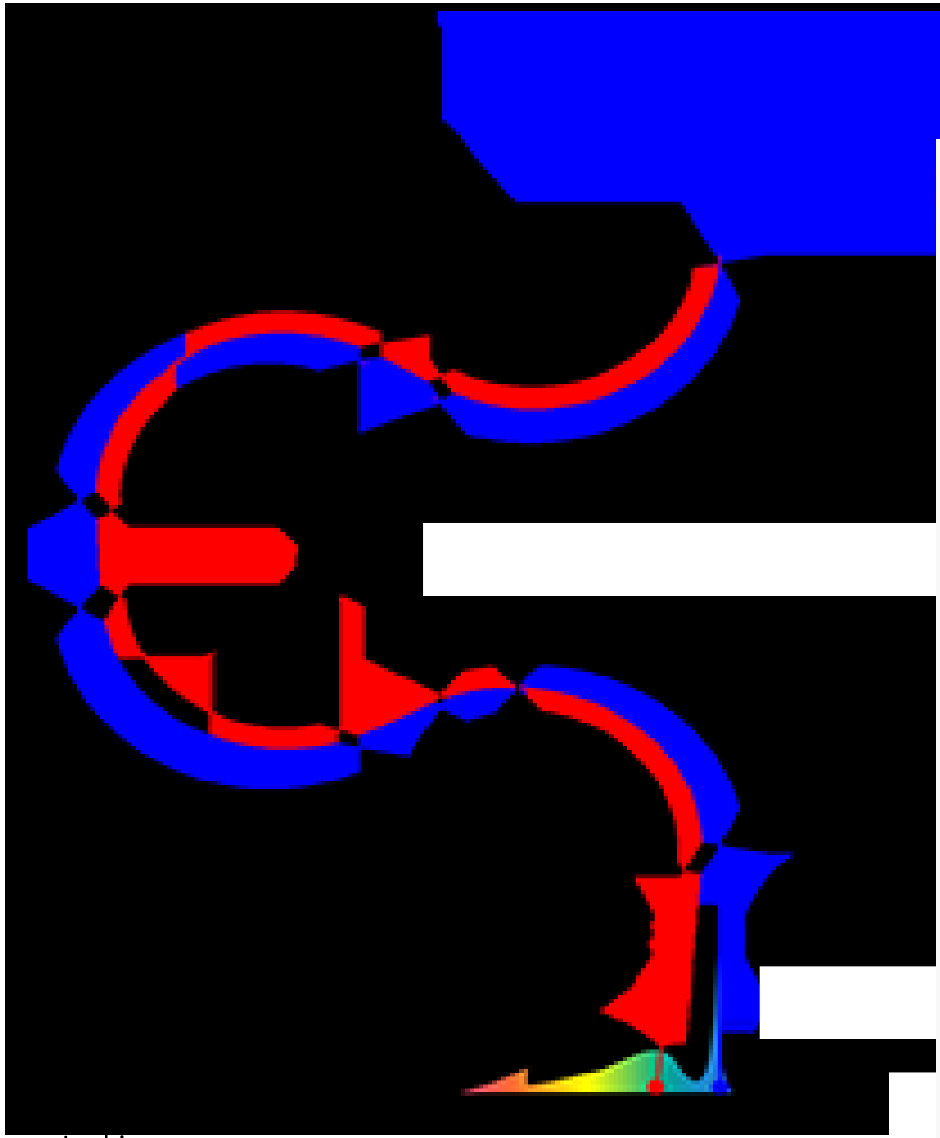
Energy filter

- Elastic electron scattering
- Inelastic electron scattering
- In column (Omega filter)
- Post column

Prism

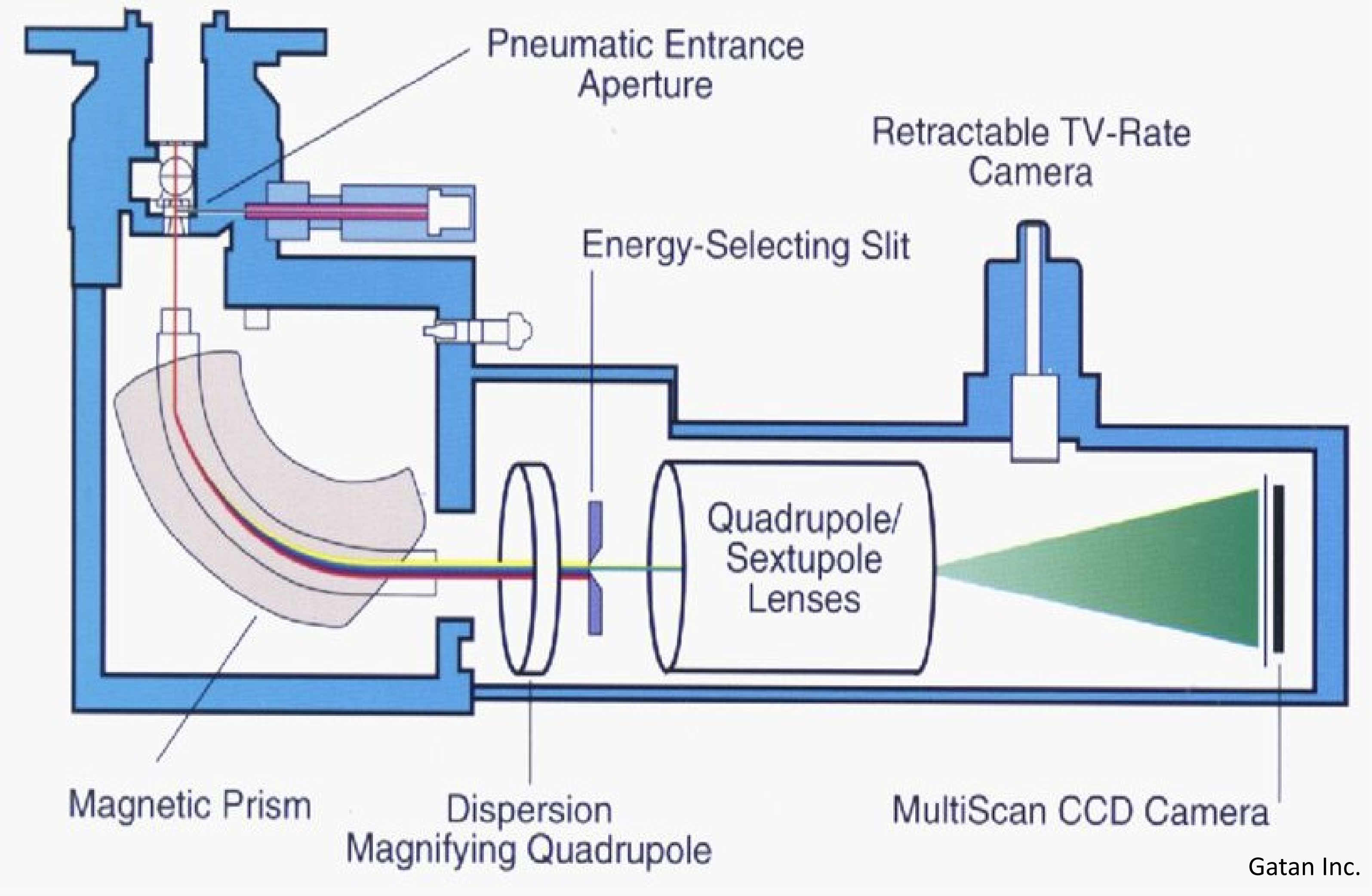


Energy filter – electromagnetic prism



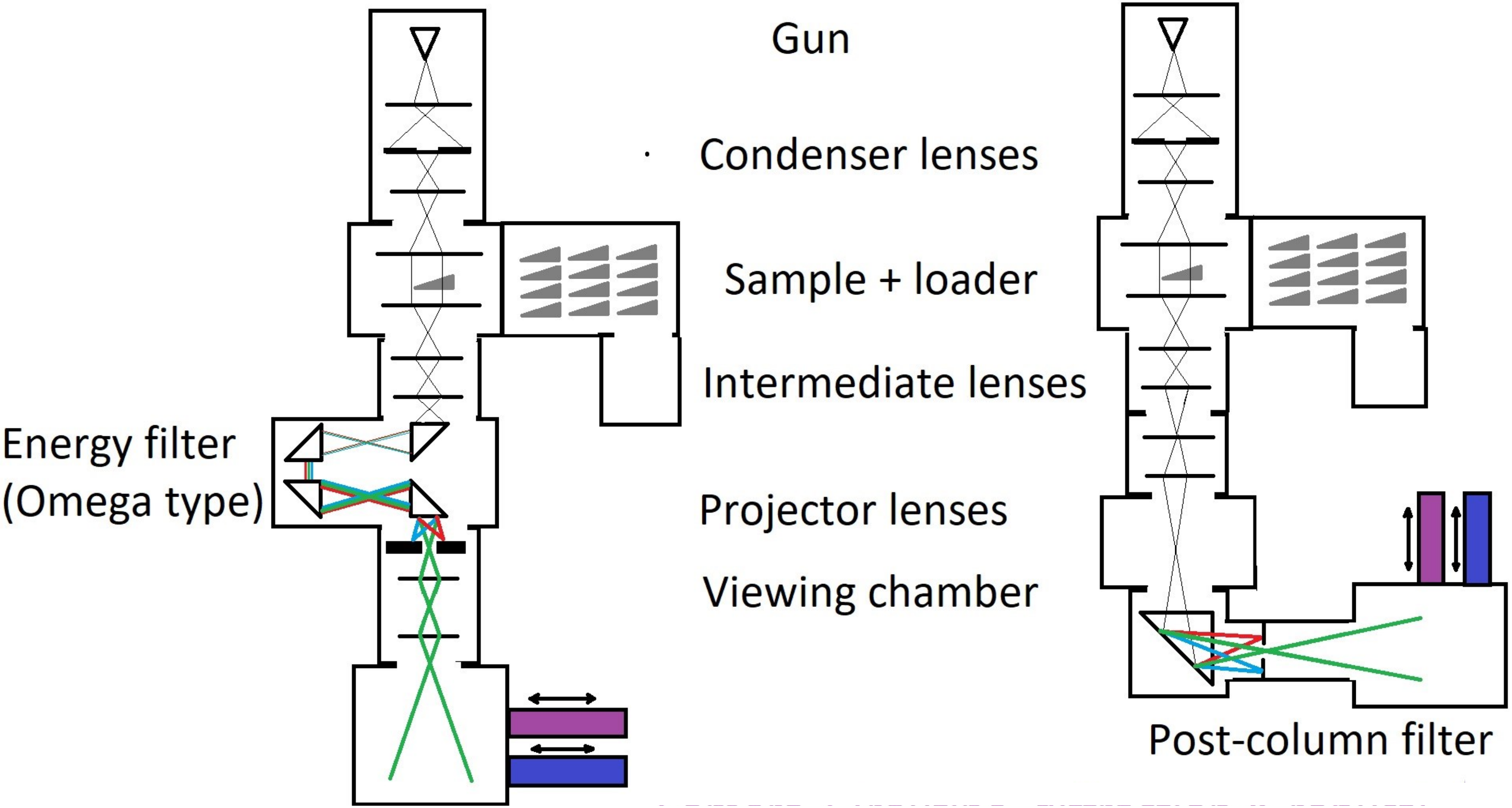
Jeol.jp

Po lumn filter



Gatan Inc.

In-column / post-column filter



Gun

Condenser lenses

Sample + loader

Intermediate lenses

Energy filter
(Omega type)

Projector lenses

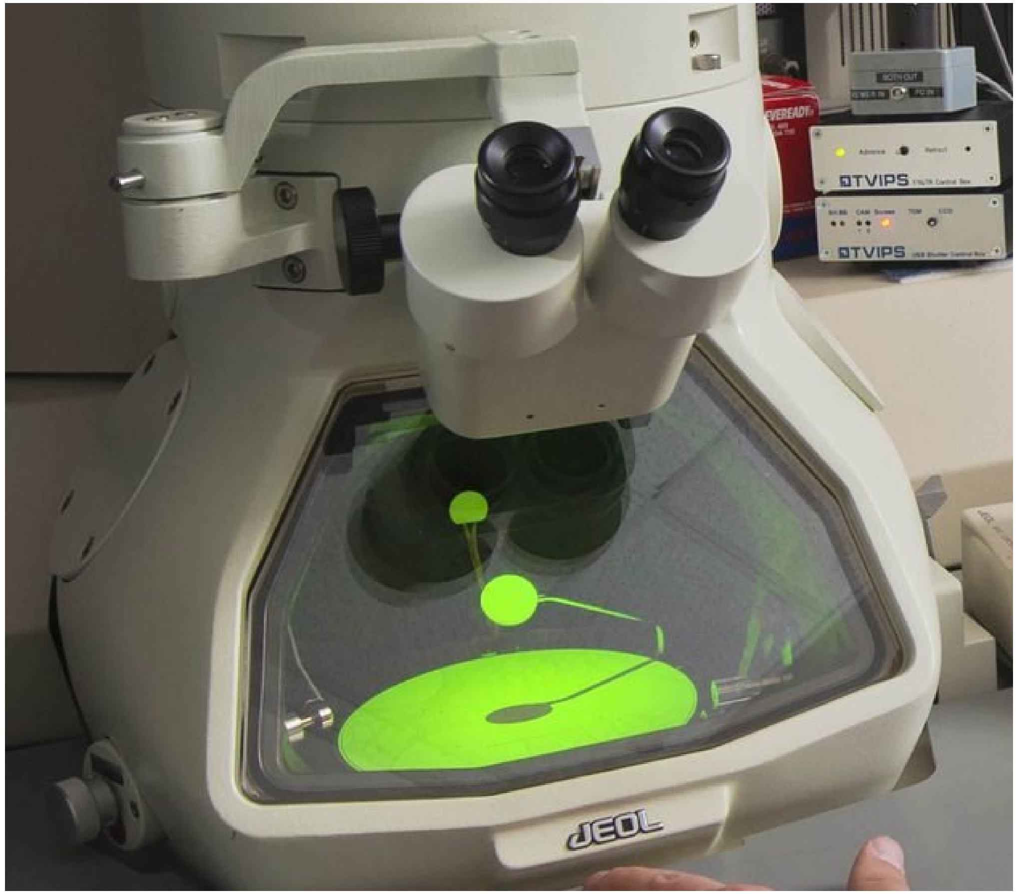
Viewing chamber

Post-column filter

thruanelectronlens.wordpress.com

Detectors

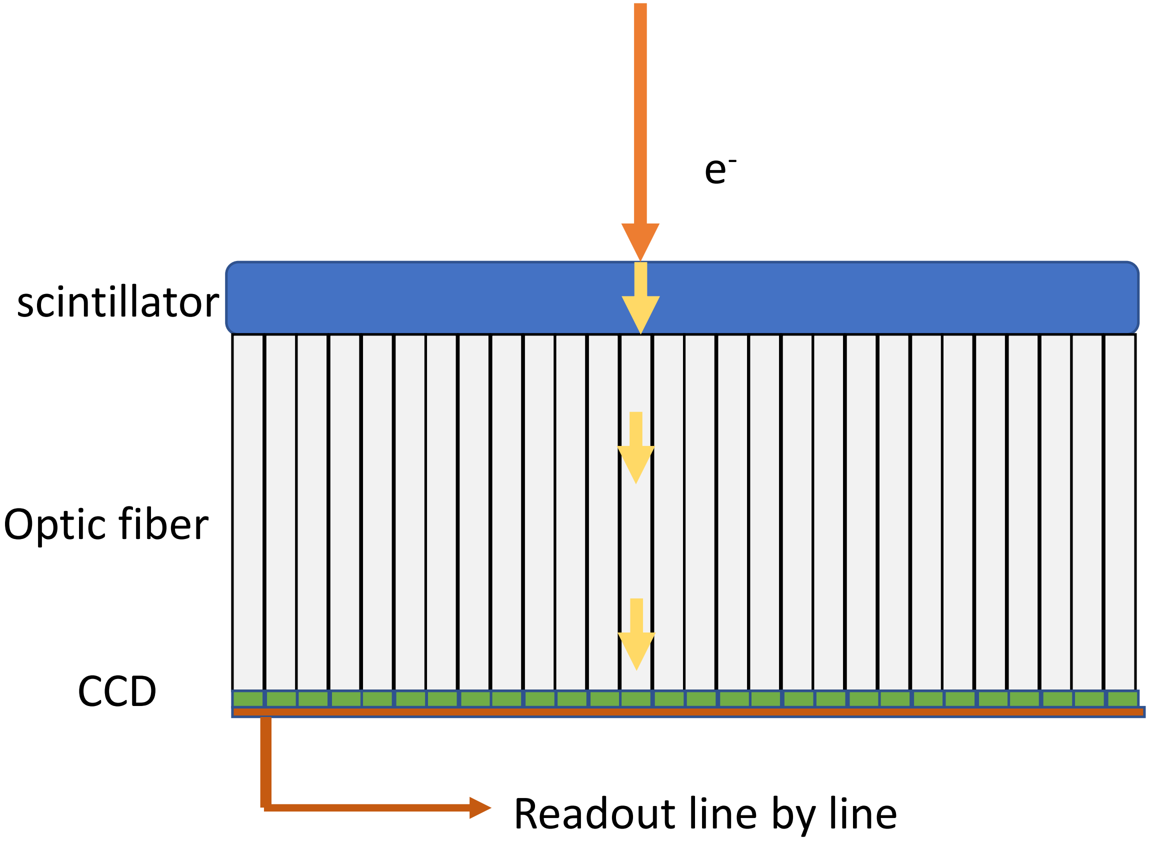
- Fluorescent screen
  - Zinc sulfide
  - Robust
- Film
  - Silver reduced from silver halide
  - Linear response
  - Digitalization of films
  - No drift correction
- CCD – charge couple device
- Direct electron detector

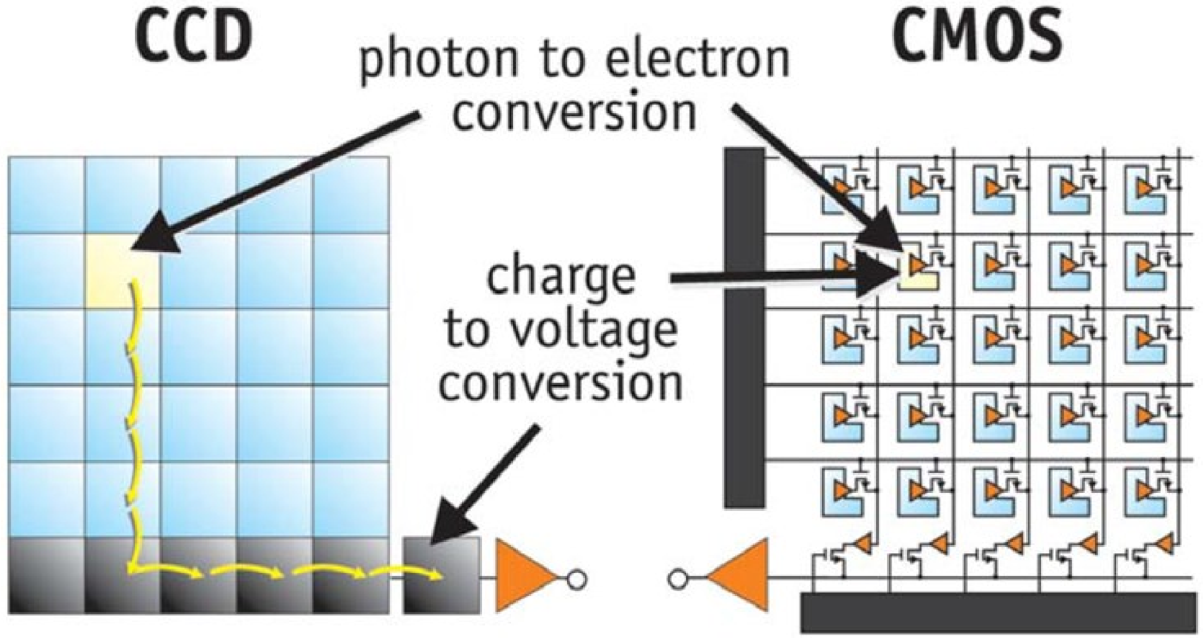


twitter.com/difluorine

CCD camera

- Convert e^- to light and detect by CCD
- Sensitive to cosmic rays
- Scattering between scintillator and fiber optics



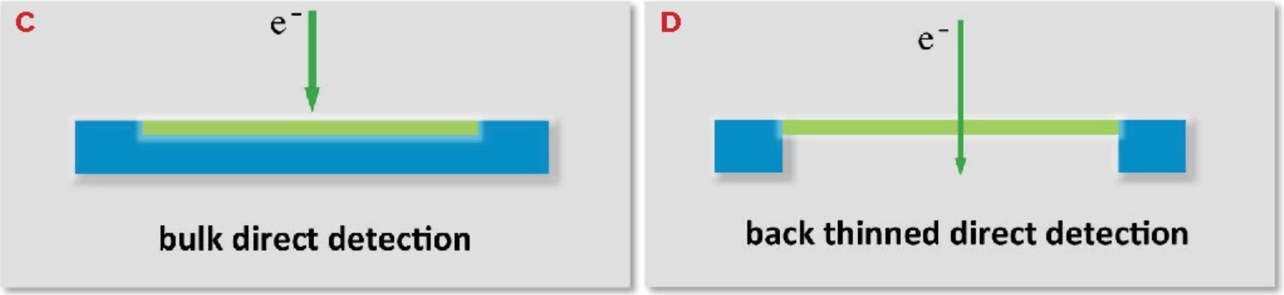


CCDs move photogenerated charge from pixel to pixel and convert it to voltage at an output node. CMOS imagers convert charge to voltage inside each pixel.

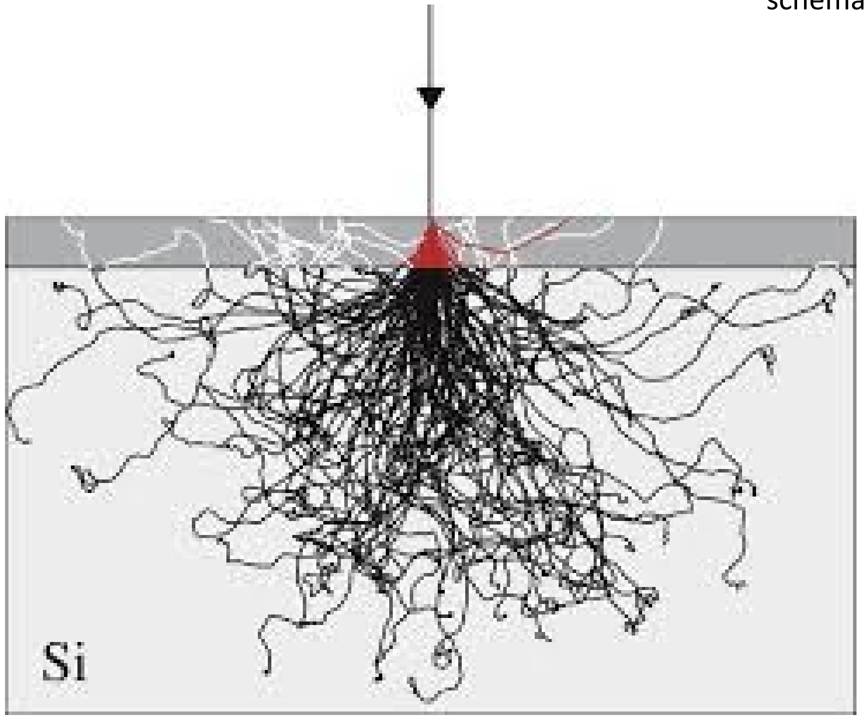
http://meroli.web.cern.ch

Direct electron detector

- Directly detects electrons (no conversion to photons)
- Non-sensitive to cosmic rays
- Fast readout (movie mode)
- Back thinning reduce e$^-$ backscattering
- Every pixel own readout electronics
- Linear (integrative) mode
- Counting mode

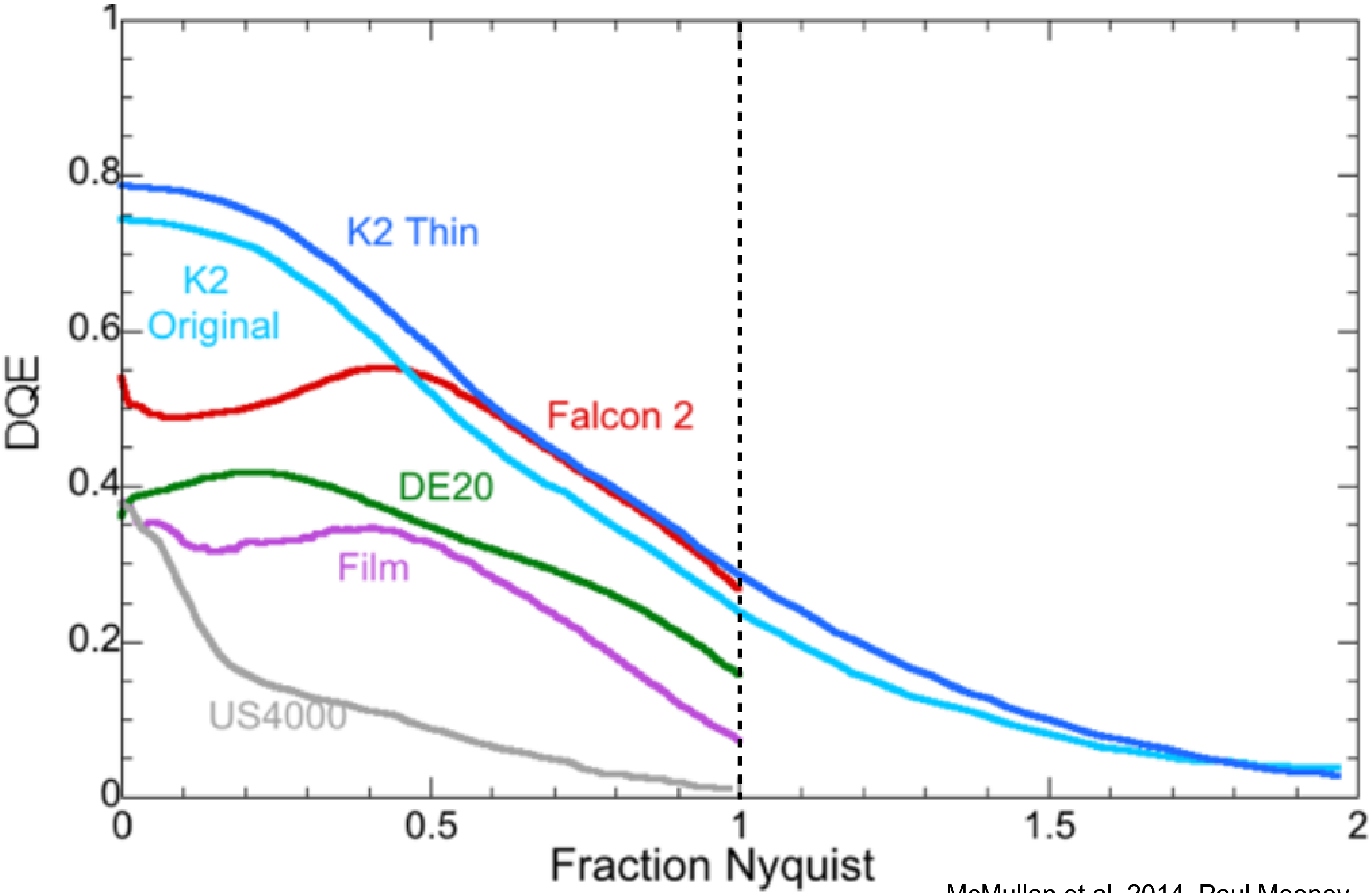


schemanticscholar.org



franklab.cpmc.columbia.edu

DQE - detective quantum efficiency



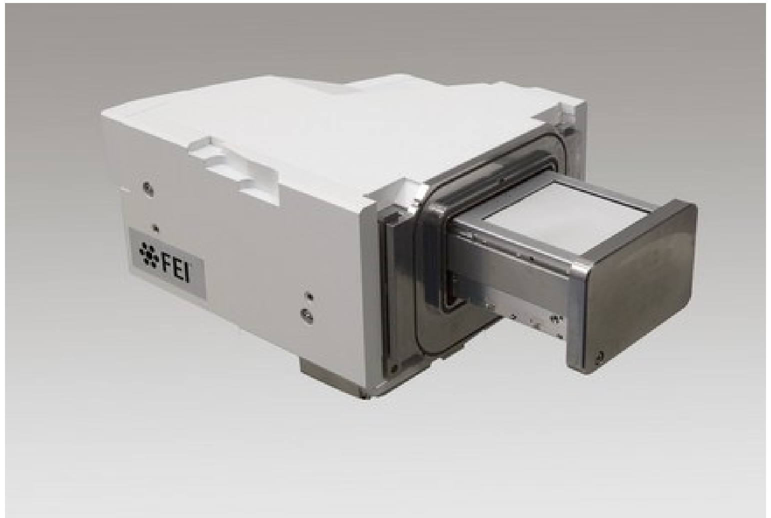
McMullan et al, 2014, Paul Mooney

Direct electron detector

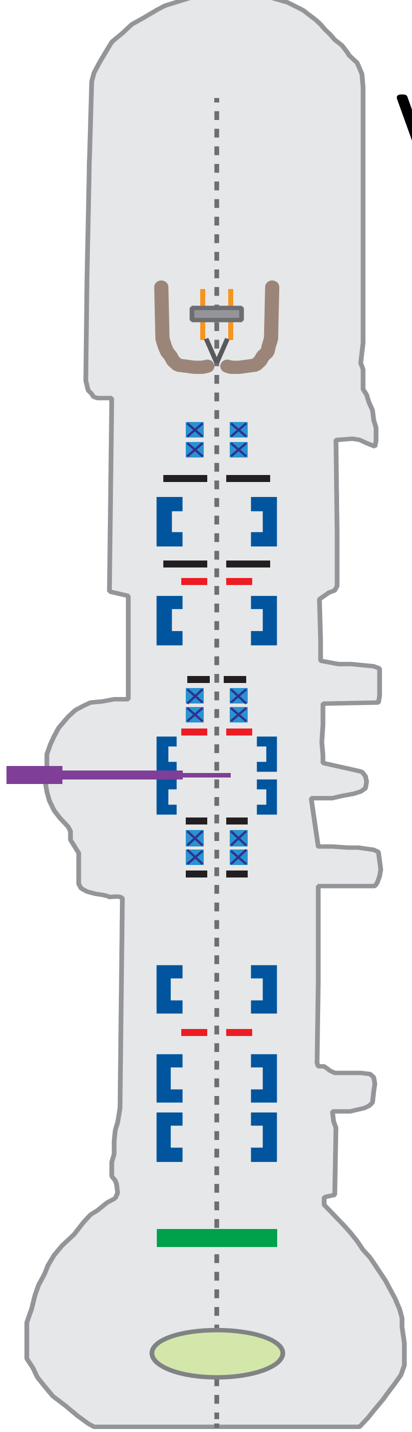
ThermoFisher – Falcon 4

Gatan K2 summit

Location in the microscope



ThermoFisher – Falcon 3

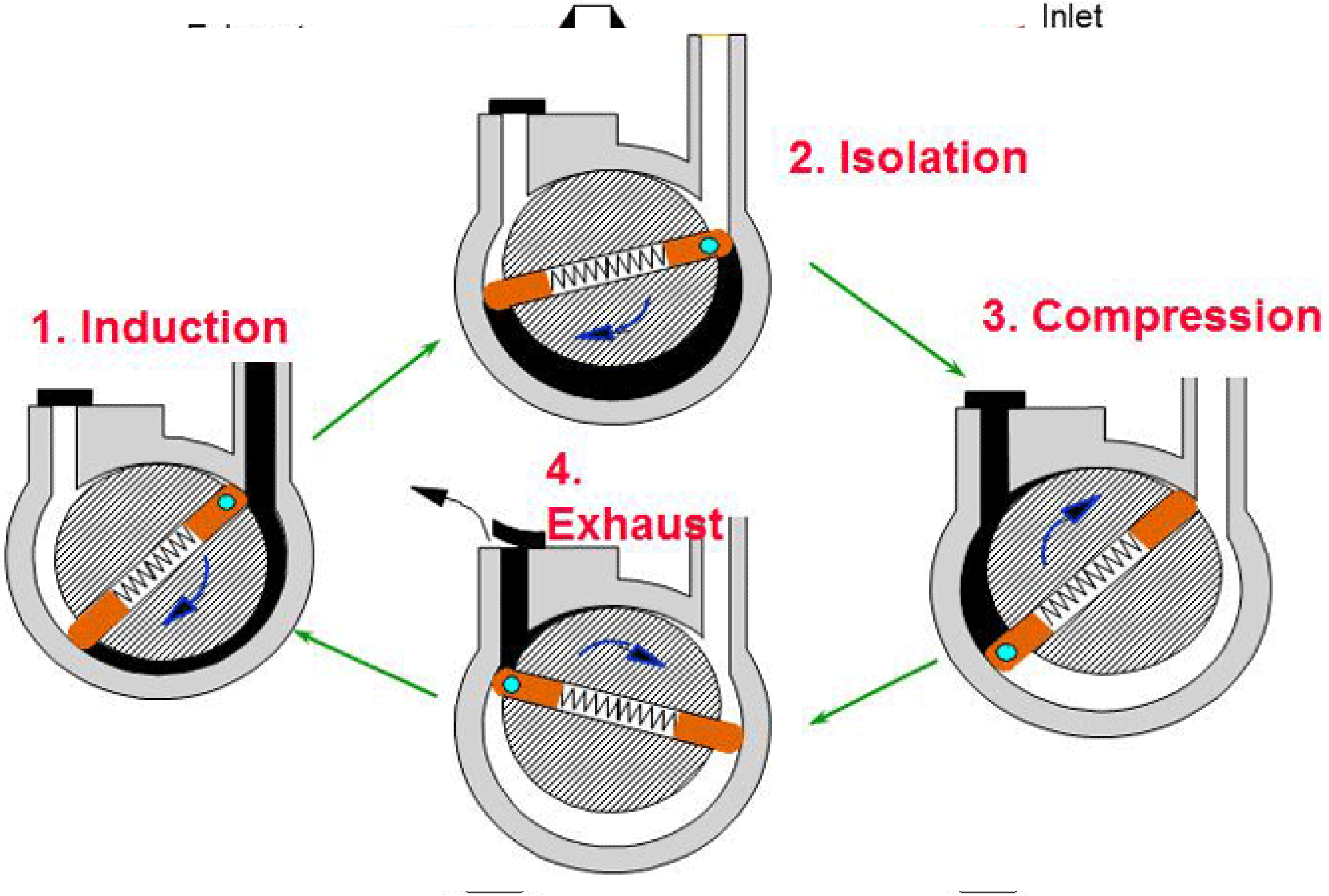


Vacuum system

- TEM requires high vacuum
- Rotary pump (low vacuum up to ~10^{-4}Pa)
- Oil diffusion pump (high vacuum ~10^{-8}Pa)
- Turbomolecular pump (high vacuum ~10^{-8}Pa)
- IGP – ion getter pumps (ultrahigh vacuum up to 10^{-9}Pa)

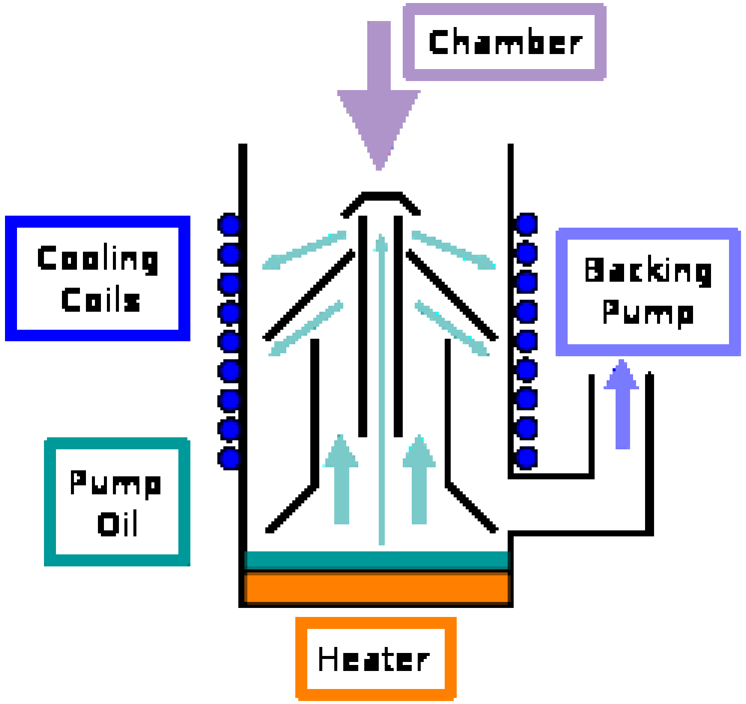
Rotary pump

- Mechanical pump
- Produce vibrations
- Low vacuum
- Used as backing pump
- Dry scroll pumps



vacaero.com

Oil diffusion pump

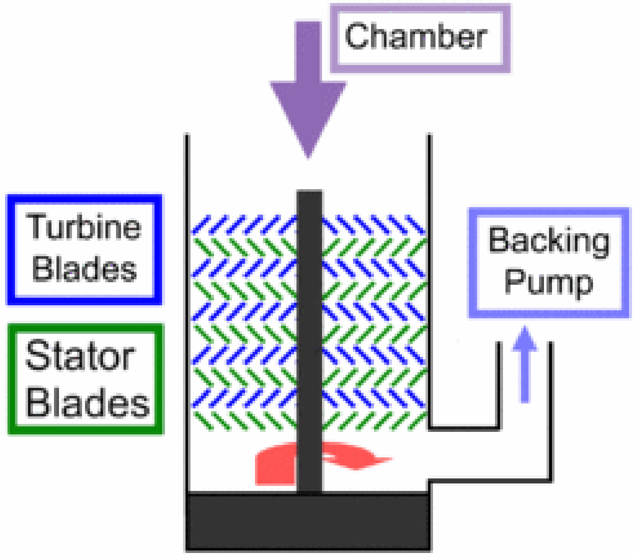


wikipedia.org

- High vacuum
- Requires intermediate vacuum
  - Need backing pump

wikipedia.org

Turbomolecular pump



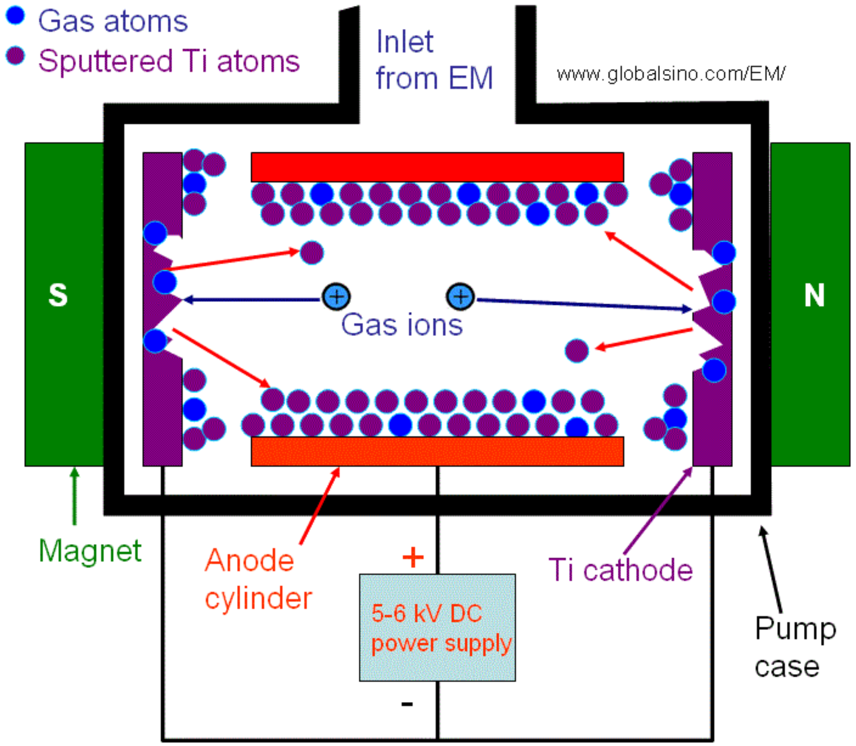
wikipedia.org

~100 000 RPM

wikipedia.org

Ion getter pump

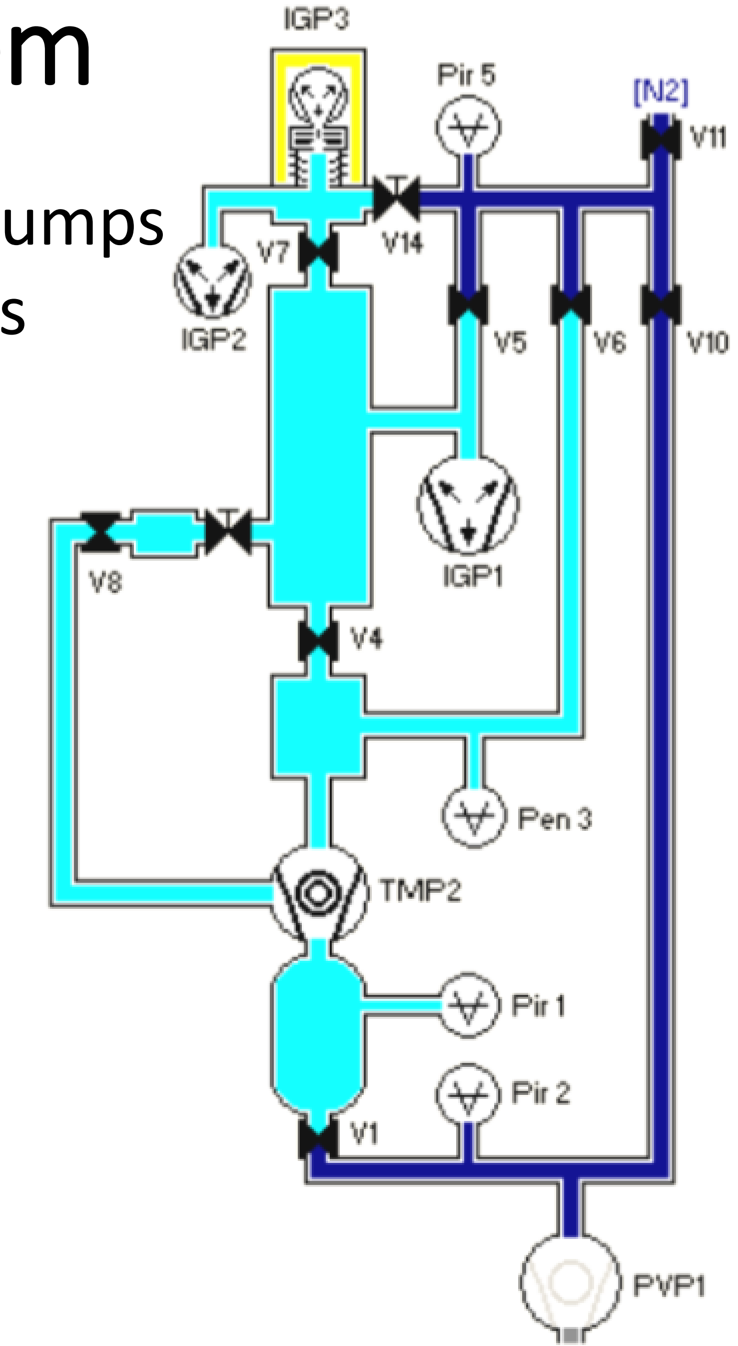
- Ultra-High vacuum
- Slow
- Requires high-vacuum
  - Turbomolecular pump backed



www.globalsino.com/EM/

Vacuum system

- Combination of pumps
- Isolated chambers
- Pressure gauges
- Buffer tank

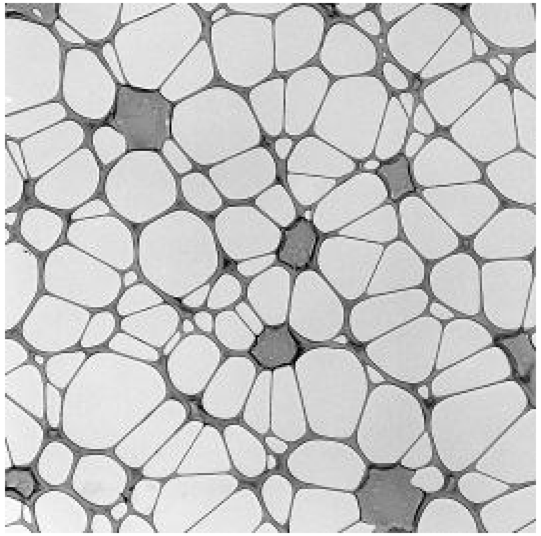


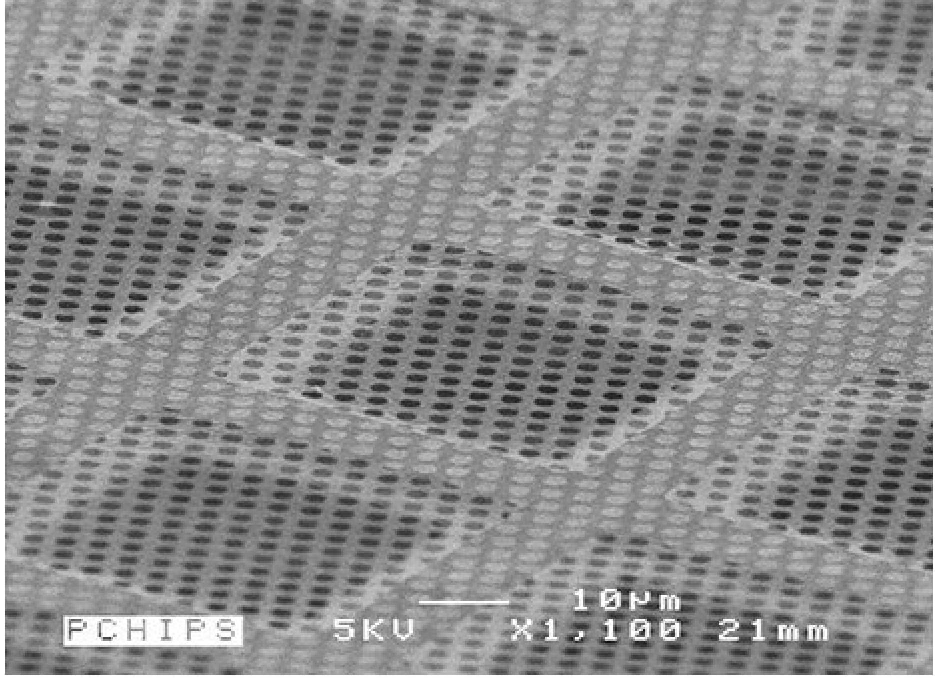
Sample preparation

- Preparation of the perfect sample to image
  - The better sample the better result
  - 1st most difficult step
  - molecular biology / microbiology / biochemistry
  - Preparative methods – proteins / protein complexes
- Vitrification
  - Embed the sample into vitreous ice
- Vitrified sample storage
  - Keep the sample vitrified

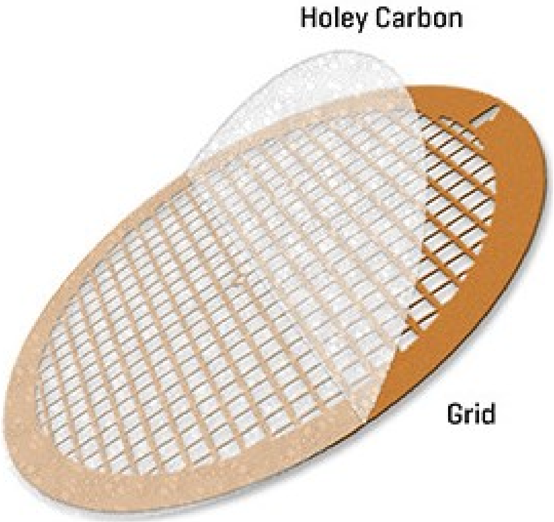
Types of EM grids

- EM grids
  - Copper
  - Gold
- Grid coating layer
  - Carbon coated
    - Lacey carbon
    - Holey carbon
    - Carbon coated holey carbon
  - Holey Gold (UltrAuFoil)
- Hole parameters
  - Hole shape – circular, hexagonal
  - Hole size/spacing – 2/1; 2/2; 1.3/1.6 etc….

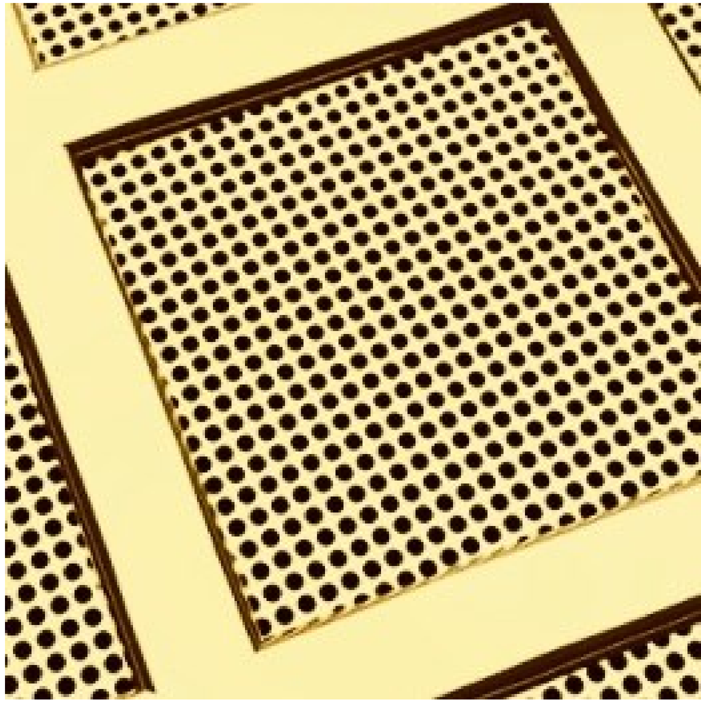

agarscientific.com



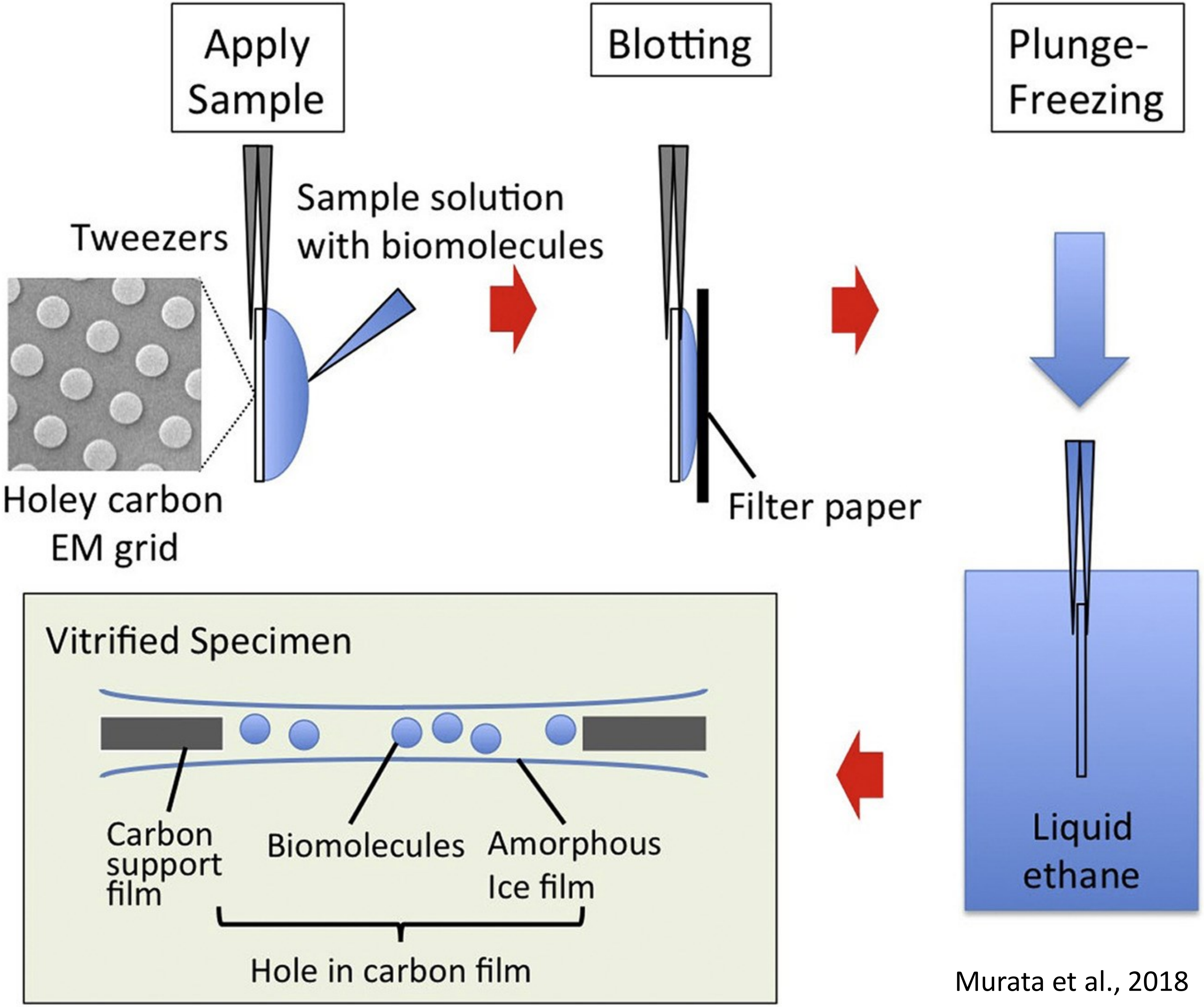
edeninstruments.com



emresolutions.com


emresolutions.com

Sample preparation - vitrification

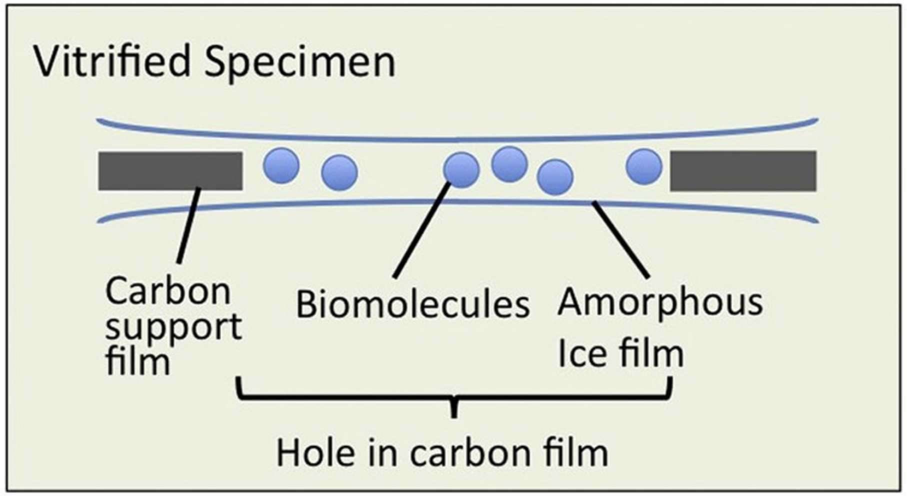


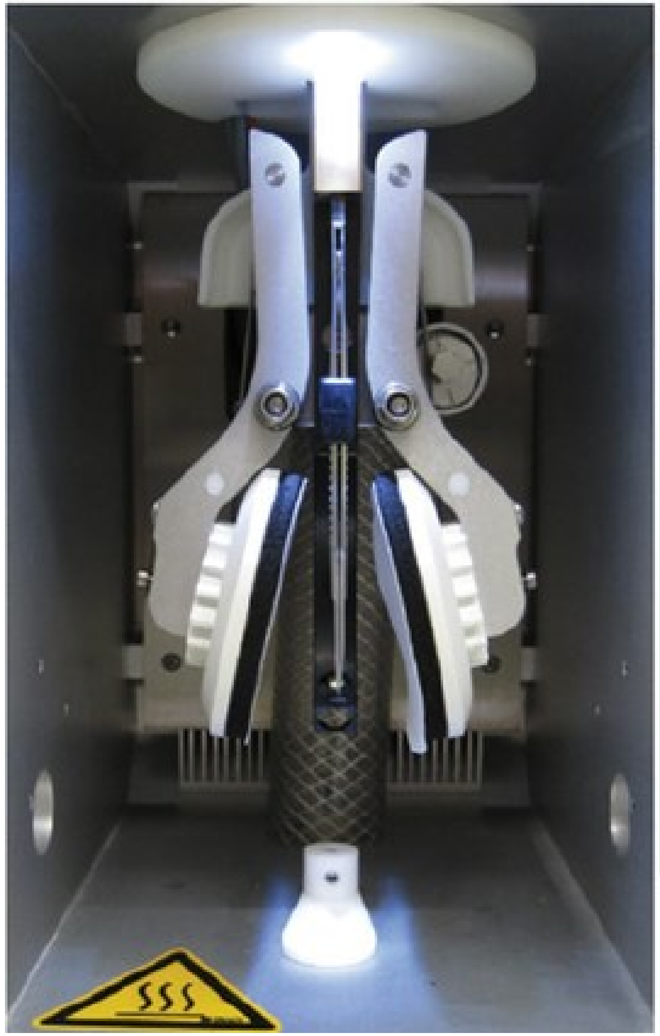
Murata et al., 2018

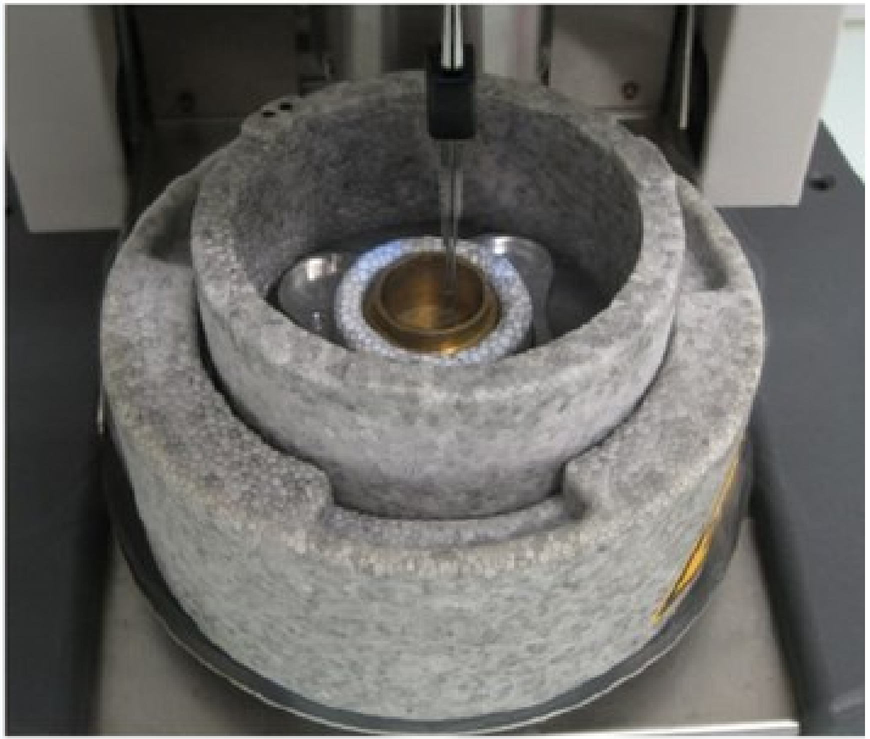
Jacque Dubochet

Vitrification - instrumentation

- Immobilization
- Why to freeze the samples
- Vitreous ice
- Beam induced damage

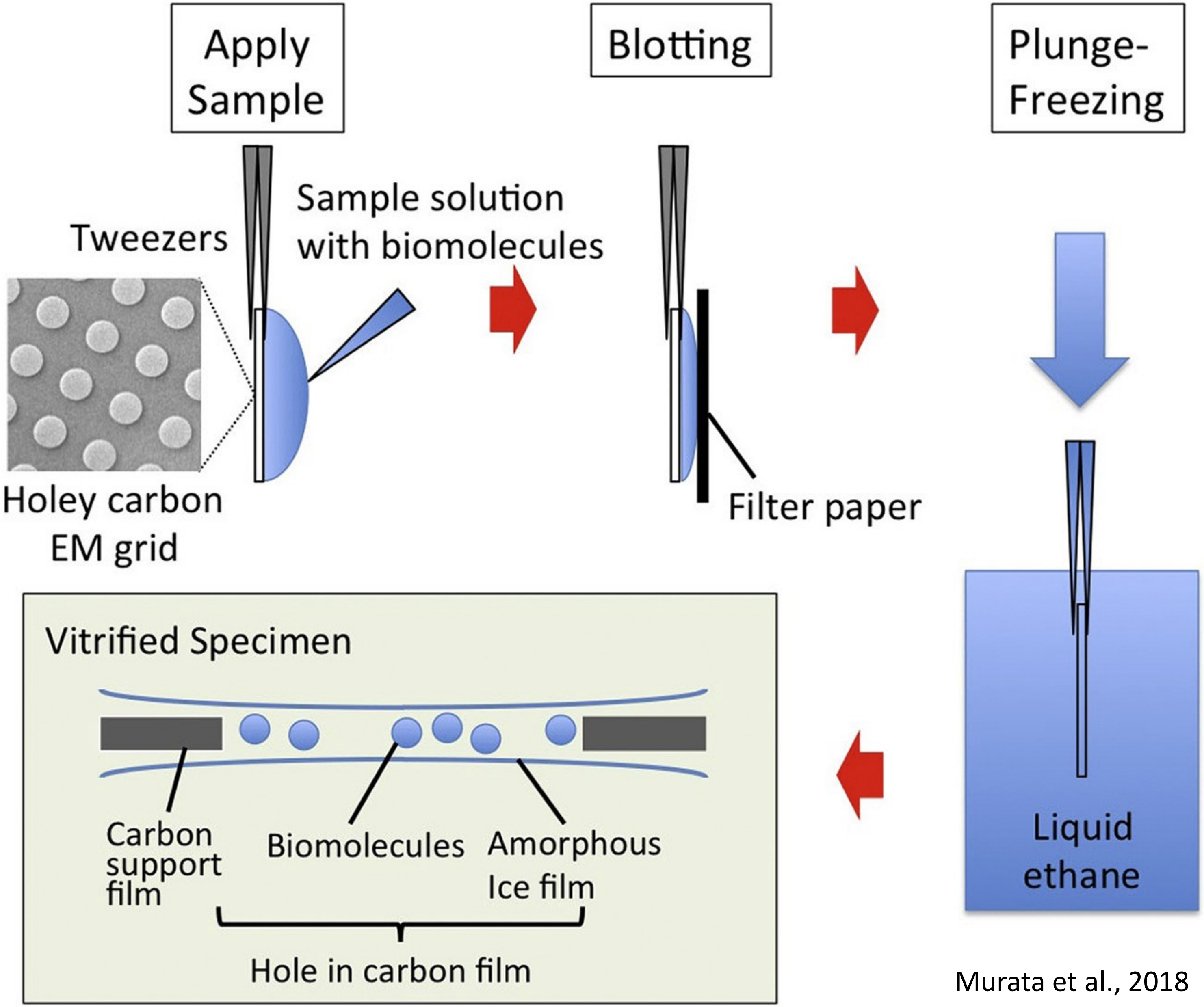






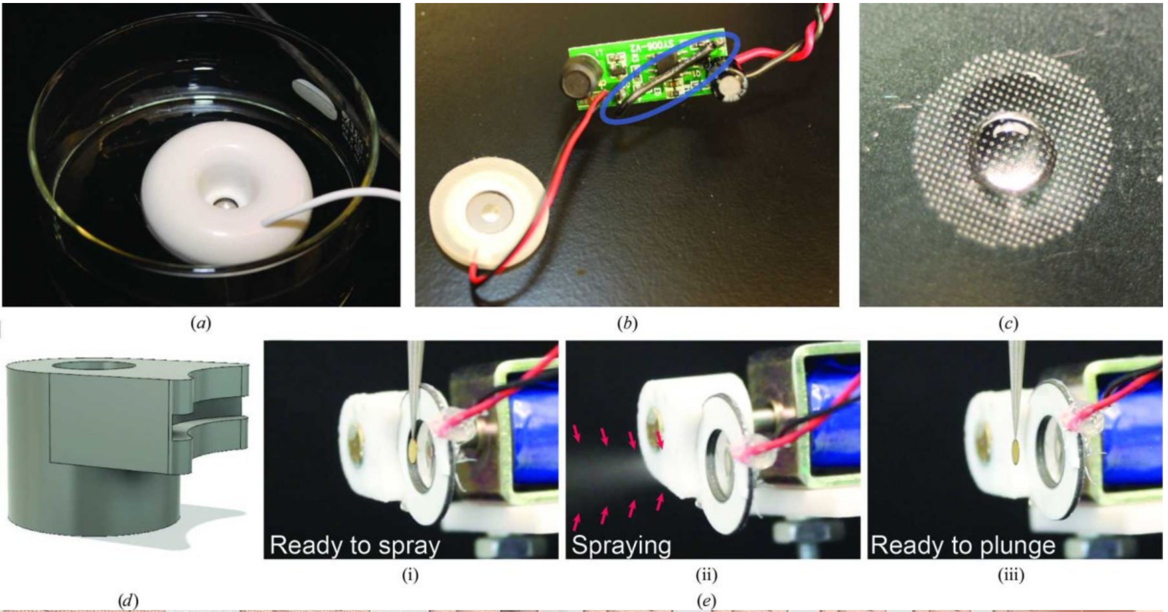
Vitrobot Mark IV

Sample preparation - challenges



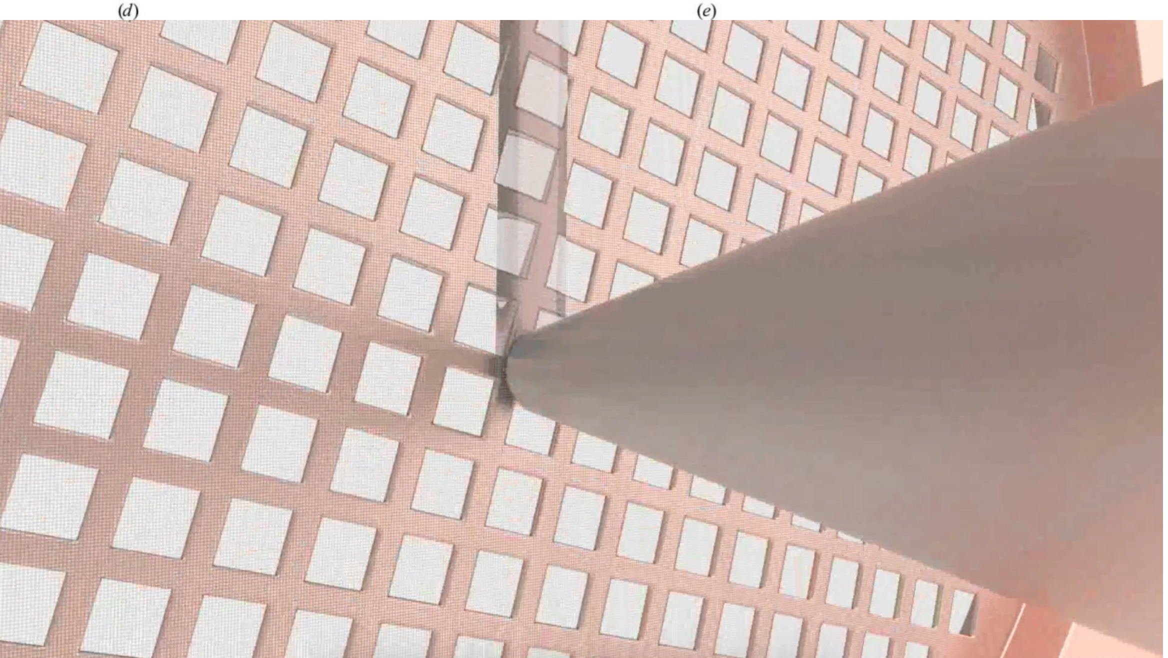
Murata et al., 2018

Spottiton, Vitrojet, *Shake-it-off*

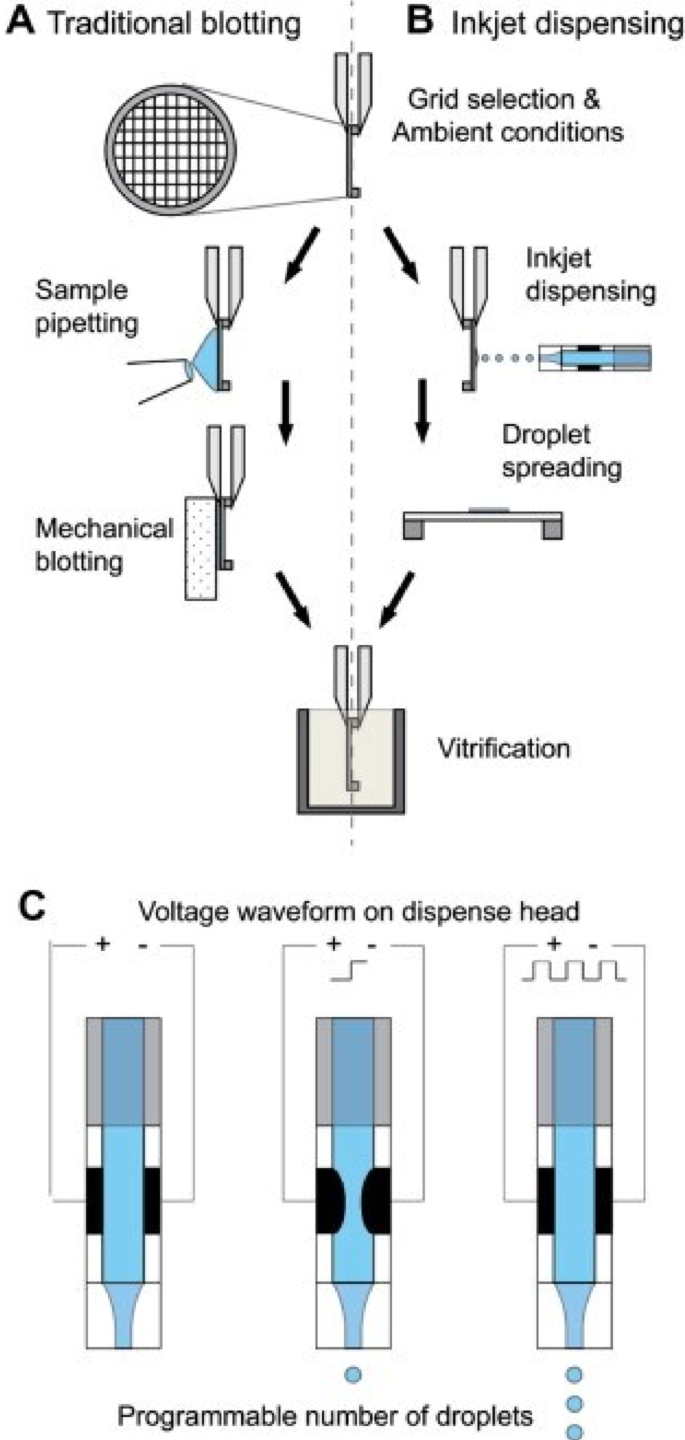


(a) (b) (c)

(d) (i) (ii) (iii) (e)



- New approaches to sample
- Preparation
- Commercial vs open source
- Not widely used yet



What we have learned…..

- Cryo-EM not a complimentary method any more
- Principles of optic parts of TEM
- Detection devices and their properties
- Non-optic parts of electron microscope
- Sample preparation

The end

ThermoFisher

Jeol