Chapter 1

Introduction

The purpose of science is to not only extend knowledge, even further bringing order into this knowledge.
-Niels Bohr

In the cells of all living beings, macromolecular machines or complexes fulfill essential tasks to keep the cell functional and productive. Such protein complexes for example are Ribosomes. They are responsible for the translation of ribonucleic acid (RNA) into proteins, where they change their structural conformation through this process [18]. Another example is the anaphase promoting complex (APC) which plays an important role during mitosis, where it initiates the anaphase [22]. In order to understand the elaborate design of a cell the exact function of those assemblies is important. To comprehend the function of a machine, structural understanding is essential. The more details are available, the larger the gain of functional knowledge will be. This leads to the task of acquiring structural information of macromolecular assemblies at the highest possible resolution.

1.1 Three Dimensional Model Determination of Macromolecular Assemblies

In order to identify the three-dimensional (3D) structure of proteins many different methods were developed to tackle the difficulties at near atomic resolutions. The two methods based on scattering are x-ray crystallography and cryo-electron microscopy (CryoEM). Both are widely used to determine structures of proteins and macromolecular complexes.

1.1.1 X-Ray Crystallography and Cryo-Electron Microscopy

The oldest and most widely spread method for structure determination is x-ray crystallography, which is a 100 years old. It requires the sample protein to form crystals, which is a poorly understood process and mostly empirical. Formation of crystals itself is a very selective purification step, which allows only identical molecules ideally in the same conformational state, to form the crystal. A crystal diffracts light and the diffraction pattern reflects the inner structure of the crystal and the structure of the molecules forming the crystal. The wavelength of light is not short enough to resolve atomic details. X-rays, which offer a much shorter wavelength than light, are used to generate the diffraction patterns of protein crystals. These diffraction patterns are then used to calculate the 3D structure of the proteins forming the crystal. A problem during the reconstruction of the 3D structure is the missing phase information from the diffraction pattern.

X-ray crystallography achieves very high resolution. Structures were solved with less than a 0.5 Ångstrom resolution [63]. A drawback of this approach is the high amount of purified sample required to screen for crystallization conditions and to optimize the crystal quality. Furthermore, it is very intricate to generate crystals from large molecules or from molecules which exist in different conformational states.

A newer approach for the acquisition of 3D information of proteins is cryo-electron microscopy (CryoEM) combined with single particle image processing. Electron microscopy (EM) is somehow similar to light microscopy. Instead of using light, which does not provide an appropriate wavelength for atomic detail, electrons are used. To alter the beam path magnetic lenses are used instead of lenses made of glass. The instrument itself is termed transmission electron microscope (TEM), because the sample is irradiated with an electron beam, creating a two-dimensional (2D) projection of the specimen. The sample protein is placed on a copper grid in a film of vitrified ice. The ice is holding the proteins in random orientations in place. Due to the low signal to noise ratio (SNR), the quality of these projection images is a challenge for subsequent image processing steps. The computational steps are required to determine the 3D structure which was lost by projection the proteins into 2D space.

The advantage of this method is that it can visualize protein complexes larger than 125 kDa. There is no crystallization required and the amount of sample needed is very small. Furthermore, heterogeneous states of the same protein are allowed to be present in the same sample. These states can be sorted and classified during the later computational part of the method. This will give insight into the conformational changes of a macromolecular complex. This enables dynamic representation of a molecule at work [18]. CryoEM faces difficulties dealing with proteins smaller than 125 kDa.

To visualize the structure of macromolecular complexes, which are fairly large (e.g. ribosomes with a molecular weight of 2.6 MDa) present in heterogeneous conformations and different moving states, single particle CryoEM is the most promising technique.

Table 1.1: Comparison of cryo-electron microscopy and x-ray crystallography

Feature / Method	**Single Particle CryoEM**	**X-ray**
Sample Size	$>$ 125 kDa	$<$ 100 kDa
Max. obtained Resolution	3.1 Å	0.48 Å
Arbitrary Conformations	possible	impossible
Preparation	freezing	crystallization process
Instrument	TEM	x-ray source, up to synchrotron
Structures solved (June 10th, 2014)	1904	100843

1.2 Electron Microscopy

The natural way for a human being to get insight into the structure of an object is to study images of this object. Images are intuitively easier to comprehend than diffraction patterns and they do not suffer from missing phase information. The first approach to visualize very small objects was light microscopy. But the resolution r is limited by the wavelength λ of the light due to Abbe's Law [1]:

$$r = \frac{\lambda}{2n\sin(\alpha)} \tag{1.1}$$

n is the refractive index and α the opening angle of the objective. Modern techniques in light microscopy (STED, STORM, PALM,...) are able to localize different fluorophores at high resolution, thus circumventing Abbes's Law. The stimulated emission depletion (STED) method developed in 1994 by Prof. Stefan W. Hell [30], reaches resolution levels of 24Å$\pm$3Å[82], which is still far behind the resolutions of other imaging techniques.

To be able to get a better understanding of the atomic details of cells and their interior parts, the resolution has to be increased. According to equation 1.1 this can be achieved by decreasing the wavelength of the imaging wave. Not only light is of wave nature, there are other objects like electrons that can be seen as wave objects, defined by the deBroglie wavelength:

$$\lambda = \frac{h\sqrt{1-(v/c)^2}}{mv}, \tag{1.2}$$

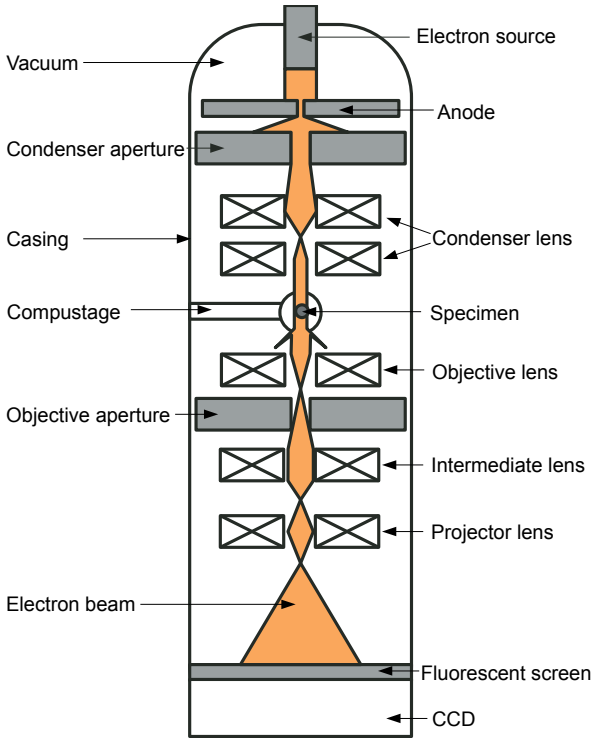

Figure 1.1: Schematic representation of a transmission electron microscope. The electron beam (orange), the apertures (gray boxes) and lenses (crossed boxes), as well as the electron source, fluorescent screen and the charge coupled device

where λ is the wavelength, m the mass of an electron, v its velocity and c the speed of light. This thought lead to the electron microscope; an instrument which uses electrons to image a sample. The advantage of electrons over photons is their much shorter wavelength, which depends on their velocity. Since the wavelength is limiting the achievable resolution, the transmission electron microscope (TEM) achieves a much higher, nearly atomic, resolution. For example on symmetrical viruses, e.g. a cytoplasmic polyhedrosis virus, a resolution of 3.1 Å was reached using a TEM [83].

1.2.1 The Transmission Electron Microscope

The instrument used to visualize objects at near atomic resolution using electron waves is the TEM. It was invented by Ernst Ruska and Max Knoll in 1931 [36]. The first TEM magnified specimen by a factor of 31; far below optical microscopes of this time. Only two years later an improved version was constructed, which was able to go beyond the optical resolution limit.

The electron microscope uses a coherent beam of electrons, generated by an electron source. The most coherent beam is currently generated by a field emission gun (FEG)

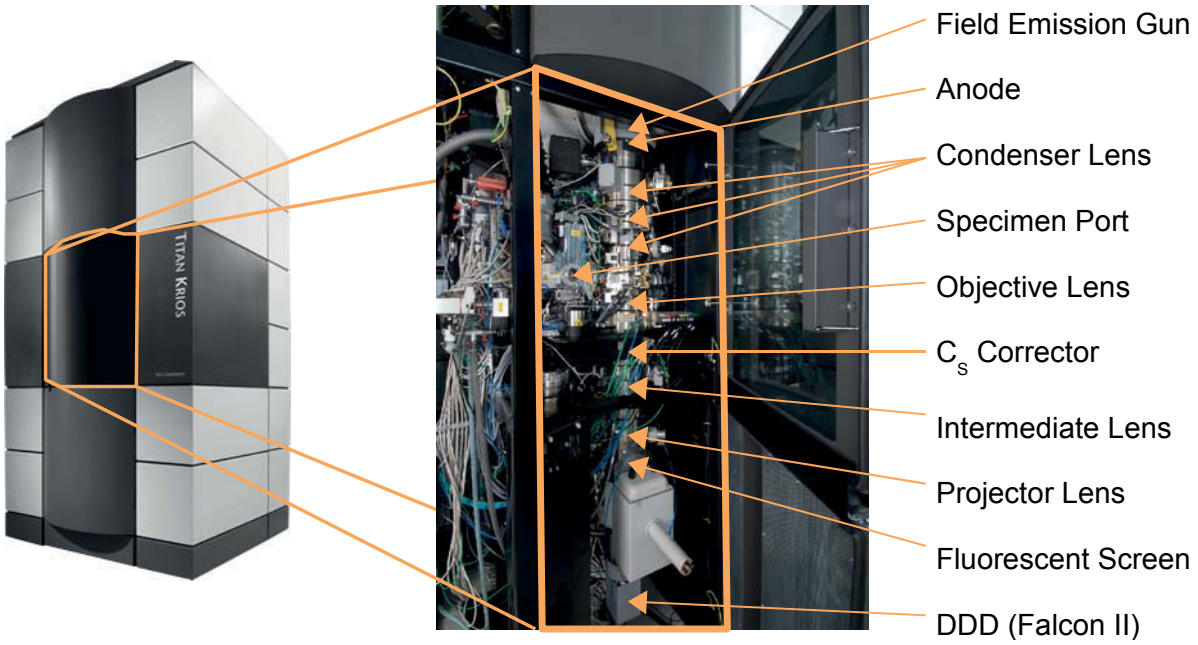

Figure 1.2: C_s corrected Titan Krios with a direct detection device Falcon II, installed at the 3D CryoEM department of the Max-Planck institute for biophysical chemistry in Göttingen.

using a strong enough extraction voltage to enable the emission of field electrons into the vacuum.

Electrons are highly accelerated in an electrostatic field with an acceleration voltage of up to 500kV. The electron beam created this way can be compared to the light beam in an light microscope. The beam is modified by different lens systems and restricted by apertures. In case of the electron beam, these lenses are strong toroid magnetic fields, which are focusing the beam.

The schematic setup of a TEM is shown in figure 1.1. The first set of lenses passed by the beam are the condenser lenses to adjust the illumination of the specimen. These lenses are focusing the beam on the specimen. They are also used to define the diameter of the beam and therefore the electron dose the sample is exposed to.

The specimen, positioned in a moveable and tiltable specimen holder called compustage, is irradiated by this beam near the focal point. Inside the specimen three phenomenons simultaneously taking place. The first phenomenon is the absorption of electrons of the atomic nuclei of the sample, followed by x-ray radiation, which is a relatively rare event. The second phenomenon is electron-electron interaction as inelastic scattering of beam electrons with shell electrons of the nucleus. The third effect taking place is elastic scattering of electrons induced by the Coulomb potential of the protons in the specimen's atomic nuclei, inducing a phase shift to the electron wave. If the scattering induced

by heavy atoms is very strong, electrons may be removed from the beam, resulting in a change of teh electron wave amplitude. The same holds true for absorbed electrons.

Electrons passing through the sample and have not been scattered out of the beam are focused again by the objective lens. This creates a first intermediate, inverted image, which is later magnified multiple times. In the back focal plane of the objective lens a diffraction pattern is formed.

Next, the beam passes the intermediate lens. Here the image formed by the objective lens is magnified. The focus of this lens is either set to the back focal plain to observe the diffraction pattern or into the focus of the objective lens to form an image.

The last lens, the projector lens, magnifies the image formed by the intermediate lens one last time and projects it onto a photographic film or a fluorescent screen.

Electrons hitting the fluorescent screen or the scintillator create a flash of light at the location of the impact. This light flash is detected by a charge coupled device (CCD), which is accumulating the flash events as long as the sample is irradiated and crates a digital image.

The microscope used in the 3D Electron-Cryomicroscopy group of the Max-Planck institute for biophysical chemistry in Göttingen, is a *state-of-the-art* instrument for life science. As shown in figure 1.2 there are some differences to a standard TEM. It uses a FEG, which is not a standard device on a microscope. Furthermore, it is equipped with a C_s-corrector that removes the spherical aberration of the electromagnetic lenses of a microscope. A C_s-corrector is usually only used in material science. It is set up with a third condenser lens for parallel illumination. The imaging device is not a CCD; instead it utilizes a direct detection device (DDD). Those devices are able to record single electrons and have a lower read-out noise compared to a CCD.

1.2.2 Image Acquisition

The human eye is only able to interpret differences in contrast as an image, meaning differences in intensities. These differences are generated by changes in the amplitude. In paragraph 1.2 it was shown that in biological specimen only very little amplitude contrast is generated due to the absence of heavy atoms in the sample. The structural information of the sample is mainly resulting from phase shifts. This section is going to explain how the phase shift is generated and translated into visible amplitude contrast.

1.2.2.1 Amplitude Contrast

In most biological samples no interaction of electrons from the electron beam with the specimen occurs. They are just passing through without generating information. In paragraph 1.2 three phenomenons are described, which contribute to image information.

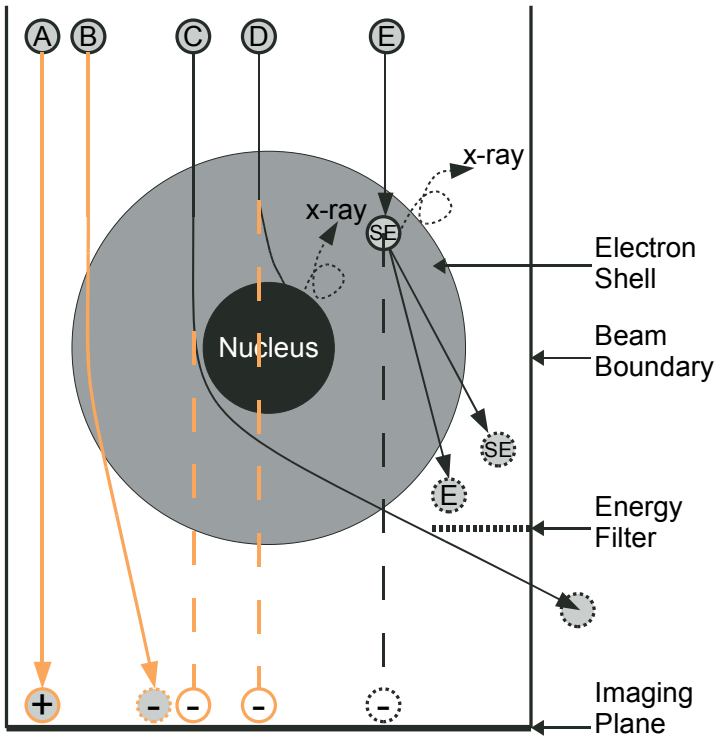

Figure 1.3: Scheme of possible interactions of beam electrons with specimen atoms. The gray circles A to E represent beam electrons, SE indicates a shell electron, x-ray indicates x-ray radiation emitted as Bremsstrahlung. Gray circles labeled with + are indicating positive intensity on the imagine plane, whereas white circles labeled with − are indicating missing intensity. Electron A does not interact with the sample. This results in positive intensity. Electron B is elastically scattered by the Coulomb force which is induced by the nucleus. This would result in false positive intensity. However, due to a phase shift it results in negative intensity. Electron C is heavily scattered and removed from the beam, which results in negative intensity. Electron D is absorbed by the nucleus and removed from the beam resulting in negative intensity. Electron E collides with a shell electron SE of the electron shell of the atom. In this process known as inelastic scattering energy from E is transferred to SE. Both electrons change their direction and speed. Thus, E and SE are slower than beam electrons and can ba removed from the beam using an energy filter to prevent origination noise, resulting in false negative intensity.

The first phenomenon, the absorption, occurs rarely and produces x-ray radiation as Bremsstrahlung which creates background noise, see D in figure 1.3. The second effect, the inelastic scattering of electrons also creates background noise due to two electrons with energy loss. Technically these electrons can be dealt with using an energy filter or a C_c-corrector, see E in figure 1.3. The phenomenon of electron scattering is what creates

most of the image information, see B and C in figure 1.3. It is induced by the Coulomb force between beam electrons and the nuclei of the sample. The Coulomb force is defined as:

$$F = \frac{1}{4\pi\epsilon_0}\frac{e_0^2 \cdot Z}{r^2}, \tag{1.3}$$

where ϵ_0 is the vacuum permittivity with a value of $8.854 \cdot 10^{-12}\frac{F}{m}$, e is the elementary charge constant with a value of $1.602 \cdot 10^{-19}C$, Z is the number of protons in the sample nuclei (which is equal to its atomic number) and r is the distance between beam electron and the center of the nucleus.

As one can see the Coulomb force increases if the distance between nucleus and electron gets smaller or when the atomic number of the specimen nucleus increases. Therefore, heavily scattered electrons are more likely for heavier atoms in the sample and the more dense the atoms in the specimen are. In figure 1.3 all five described phenomenons are visualized.

Biological samples like proteins, are mostly composed of light atoms like carbon, nitrogen and hydrogen, so the strong scatter effect (C in figure 1.3), and absorption (D in figure1.3), will occur rarely and only contribute to around 5-15% to the amplitude contrast in the image.

1.2.2.2 Phase Contrast

The last paragraph clarified that the amplitude contrast generated by a biological sample in EM is neglectable. This leads to the phase contrast and how it can be interpreted.

The important contrast in EM is generated by the wave nature of electrons, see B from figure 1.3, which induces a phase shift to the electron wave. To explain the phase contrast a biological sample is considered as weak phase object [17].

The initial electron wave ψ_0 can be described as:

$$\psi_0 = a_0 \sin(\phi_0), \tag{1.4}$$

where a_0 is the amplitude and ϕ_0 is the phase. Passing through the specimen a phase shift ϕ_s is induced, creating a resulting exit wave ψ_r:

$$\psi_r = a_0 \sin(\phi_0 + \phi_s). \tag{1.5}$$

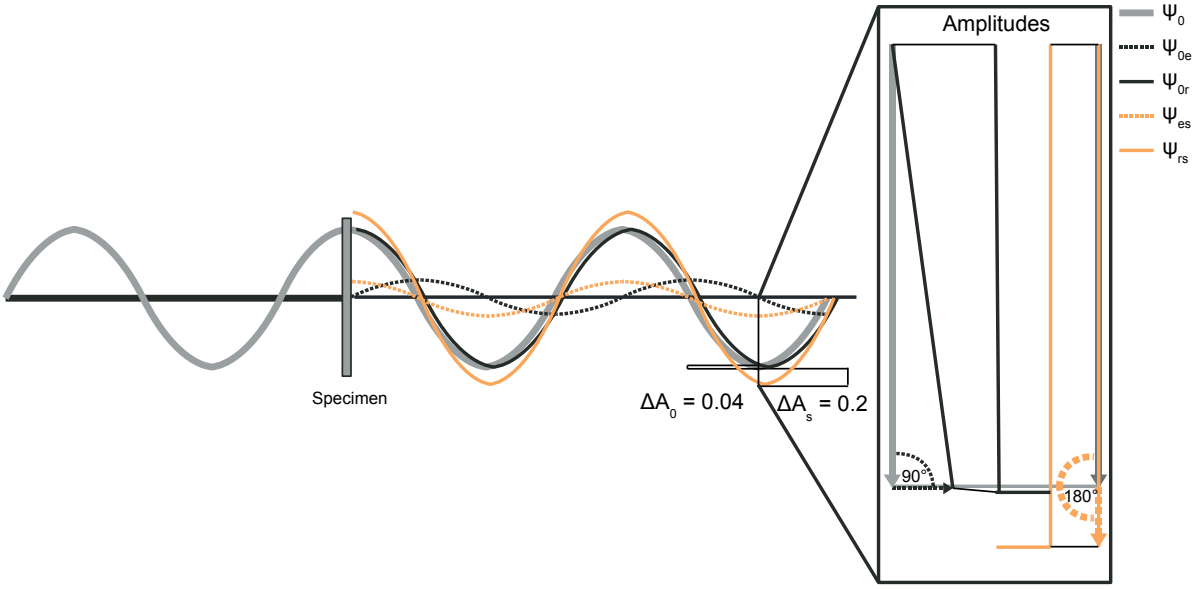

Figure 1.4: Schematic of phase shift to amplitude change. The gray curve depicts the incoming wave ψ_0. On the gray bar, symbolizing the specimen, ψ_0 is phase shifted by $\frac{\pi}{2}$ leading to the dark dotted wave ψ_{0e}. Combining of ψ_0 and ψ_{0e} leads to the resulting wave ψ_{0r} with an amplitude difference in regard to ψ_0 of ΔA_0. If another phase shift is induced, the emitted signal from the specimen would be the orange dotted wave ψ_{es} which would lead to the shifted resulting wave ψ_{rs} (orange). The amplitude difference to ψ_0 is ΔA_s. On the right the amplitude differences ΔA_0 and ΔA_s are visualized as arrow diagrams. With a phase shift $\frac{\pi}{2}$ (dark arrow) ΔA_0 is very small. An additional phase shift of $\frac{\pi}{2}$ lets the amplitude differences ΔA_s get larger (orange arrow).

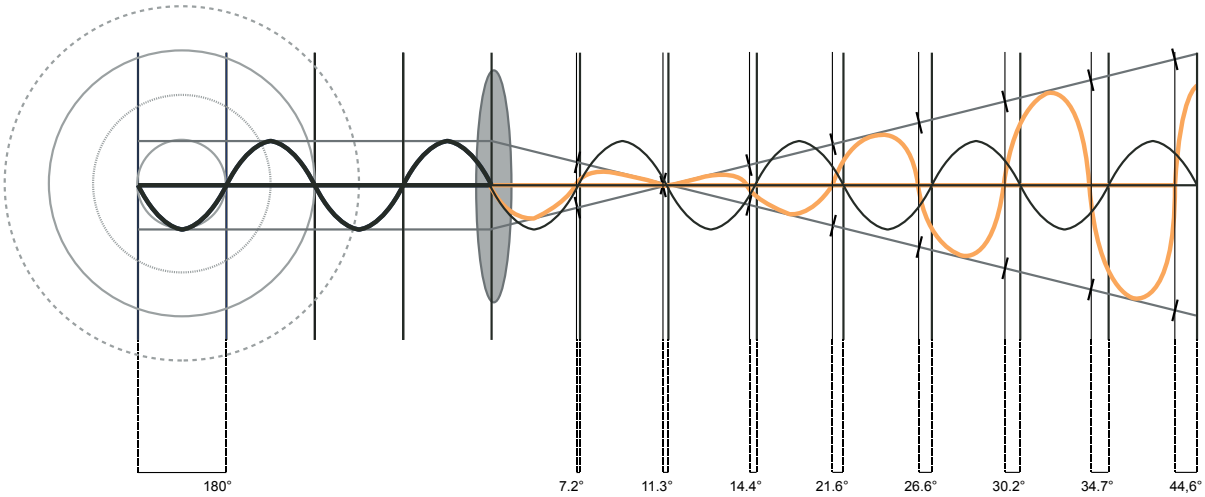

Figure 1.5: Schematic of how defocus induces phase shift. The dark wave is the non shifted incident wave. The orange wave represents the shifted wave. The gray ellipse symbolizes the lens. The phase-shift is shown at each zero crossing. The different runtimes of the waves induce a phase shift. The thin solid gray circles are wave hill, zero crossing and wave valley in this order.

Applying the sinus addition theorem [1] to equation 1.5 results in:

$$\psi_r = a_0(\sin(\phi_0)\cos(\phi_s) + \cos(\phi_0)\sin(\phi_s)). \tag{1.6}$$

Knowing the specimen is considered a weak phase object: $\phi_s << \frac{\pi}{2}$. This results in:

$$\begin{aligned} \cos(\phi_s) &\approx 1 \\ \sin(\phi_s) &\approx \phi_s \\ \psi_r &= a_0 \sin(\phi_0) + a_0\phi_s \cos(\phi_0). \end{aligned} \tag{1.7}$$

Equation 1.7 shows that ψ_r is the combination of two waves. The first summand is ψ_0. The second summand is wave ψ_e

$$\psi_e = a_0\phi_s \cos(\phi_0). \tag{1.8}$$

Using equation 1.8 and $\phi_s << \frac{\pi}{2}$ the following features of ψ_e can be deduced. First ψ_e has a very small amplitude and second ψ_e is phase shifted by $\frac{\pi}{2}$ because $\sin(\alpha) = \cos(\alpha + \frac{\pi}{2})$.

This principle is visualized in figure 1.4, represented by gray curves. The change of the resulting amplitude of the exiting wave is very small and nearly undetectable. Thus, almost no visible contrast in the resulting image is generated.

To cope with that an additional phase shift of $\frac{\pi}{2}$ has to be induced to obtain a visible change in amplitude. The orange curves, in figure 1.4, visualize the amplitudes if another phase shift of $\frac{\pi}{2}$ would have been induced to ψ_e, resulting in ψ_{es}.

To induce a phase shift in light microscopy a Zernike phase plate [84] is used. Unfortunately, for EM, no such a well working device exists. However there is another method to induce a phase shift. By defocusing the objective lens, a phase shift of variable magnitude can be added depending on the level of defocus and the spherical aberration constant. When the wave gets focused by a lens the runtime of the outer parts of the wave is increasing, resulting in a phase shift in relation to the incoming wave. In figure 1.5 this phenomenon is visualized up to a phase shift of $\frac{\pi}{4}$ or 45°.

The diagram in figure 1.5 is idealized and does not take into account the spherical aberration C_s. However, C_s is inherent to all round magnetic lenses [60] and describes the focus shift of the lens depending on the position a ray passes the lens. Taking C_s into account

[1] $\sin(\alpha + \beta) = \sin(\alpha)\cos(\beta) + \cos(\alpha)\sin(\beta)$