Breaking the diffraction limit using conical diffraction in super resolution fluorescence microscopy

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This thesis summarizes the diploma project of Roger Persson for the Master of Science degree in Physics from the Royal Institute of Technology (KTH), Stockholm, Sweden. The research was conducted at BioAxial Inc. and Institute Pasteur, Paris, under the supervision of Clément Fallet from January 2013 until July 2013. Examiner at KTH was Kjell Carlsson.

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Abstract

A new method for Super Resolution Microscopy is presented. By means of conical diffraction the excitation laser beam of a confocal microscope is modified to obtain below diffraction limit spatial frequency in the intensity profile of the point spread function. This allows data acquisition at approximately double the diffraction limited frequency. Together with a stochastic reconstruction algorithm the system provides between 2-4 times the classical diffraction limited resolution, depending on the structure of the sample. The system is built as an “add-on” feature for any standard confocal microscope and is simply plugged in as the laser source. The method is suitable for all types of fluorophores and for all types of microscope objectives, independent of numerical aperture and immersion medium. For the moment an industrial prototype of the system is being validated for “proof of concept” at Institute Pasteur in Paris.

The key part of my work with BioAxial has been the development of a simulation tool for investigation of the intensity distribution, phase and polarisation of the excitation light. The simulation tool is written on the Matlab platform. I have also contributed with experimental work, such as: alignment of the system; trouble shooting and validating measurements.
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I learned way more during those few months than I ever thought possible.

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I’m looking forward to the day I will use my very first commercially built BioAxial Super Resolution module with joy and certainty. I sincerely wish you all the progress and advancement you deserve for all the dedication and hard work you are investing in this. You will make it!

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Abbreviations

- **NA** - Numerical Aperture
- **PSF** - Point Spread Function
- **BSR** - Bioaxial Super Resolution
- **SIM** - Structured Illumination Microscopy
- **STED** - STimulated Emission Depletion
- **STORM** - STochastic Optical Reconstruction Microscopy
- **PALM** - Photo Activation Localisation Microscopy
- **PSG** - Polarisation State Generator
- **PSA** - Polarisation State Analyser
- **FWHM** - Full Width Half Maximum
- **SNR** - Signal to Noise Ratio
- **FIP** - Focal Image Plane
- **OTF** - Optical Transfer Function
- **MTF** - Modulation Transfer Function
Chapter 1

Introduction
Light microscopy has for a long time been a very important tool for biologists and other life sciences around the world. Since the invention of the microscope one has put in a lot of effort to improve the performance. What one desires is to be able to see smaller and smaller things, and more importantly, one wants to be able to distinguish between small things located a very small distance from each other. Contrary to common belief; the magnification is not the major factor. Admittedly objects appear larger in the image with a high magnification, but a higher magnification can not bring more information into the image. Resolution on the other hand is vastly more important. It is the resolution that limits the richness of detail and sharpness in the image. In the 19th century, however, Ernst Abbe [1] realised that there is an inherent physical boundary which limits how good the resolution can be for a light microscope. Due to the wave nature of light it diffracts, i.e. spreads out, when it interacts with another medium. This means for example that a single emitter of light will appear as a blurred spot when imaged, no matter how small the emitter is. The cross sectional intensity profile of an imaged spot is called the objects’ Point Spread Function (PSF). Two closely positioned emitters will appear as a single emitter if their PSF’s overlap too much.

Figure 1.1: The PFS’s of two point emitters. The dashed line indicate the total PSF from the two emitters. According to Abbe’s resolution criterion the two emitters are just resolved if the maxima of one PSF coincide with the first minima of the other.

There are a few different definitions of the resolution limit, since it is based on how you as a viewer perceive the image, but the one formulated by Abbe in 1873 [1] is the one most used when dealing with microscopy. It describes the minimum distance $\Delta x$, that two point-like emitters can be located from each other while still being separable as individual points:

$$\Delta x = \frac{\lambda}{2NA}$$

where $\lambda$ is the wave length of the light and NA is the numerical aperture of the objective. At this distance, $\Delta x$, the first minima of the PSF of one of the point emitters coincide with the maxima of the other point emitters PSF, see Figure 1.1.
Due to diffraction, i.e. the spreading of light when interacting with a medium, there is an inherent physical boundary for how good the resolution can be that is defined by the wave length, \( \lambda \), and the numerical aperture (NA) of the objective. The numerical aperture is a measure of how large the angle \( \theta \) that the objective can collect light under is, defined by:

\[
NA = n \sin \theta
\]  

(1.1)

with \( n \) for the refractive index of the medium between sample and objective. The larger the angle and hence the NA the better the resolution. A short wave length is also beneficial for the resolution. For light microscopy the wave length is however limited to the visible spectra, ranging approximately between 400-800 nm. Considering that the NA for the best objectives very rarely exceeds 1.5, we end up with a resolution of \( \Delta x \approx 135 \) nm, if the shortest visible wave length is used (400 nm). This is the best imaginable resolution with an ordinary light microscope. And it is important to remember that this assumes absolute perfect conditions. In reality the resolution will suffer from optical aberrations and misalignment of the setup etc., which means that this kind of resolution is rarely feasible.

The objectives today is highly complex units of several lenses to obtain as high NA as possible and to reduce optical aberrations. As can be seen in the definition of the NA, eq. 1.1, it is also dependent on the refractive index of the medium the sample is surrounded by. To increase the NA the use of water or oil immersion objectives has become standard procedure.

1.1 Fluorescence and confocal microscopy

Fluorescence microscopy gives a big contrast advantage as it allows labelling of specific structures in the sample. The interesting parts of the sample are the only ones giving a signal and the background is essentially black. This gives a tremendous boost in signal to noise ratio (SNR) when comparing to traditional reflection or transmission microscopy. The possibility of several colours also exists as fluorophores of different transmission wavelengths can be used. The fluorophores are attached to molecules that attach to different parts of the sample, so called markers [2].

The transitions involved for excitation and fluorescence of a typical fluorophore are described below. If the incident photon has the sufficient energy matching the electronic band gap between the ground state \( (S_0) \) and the first excited state \( (S_1) \) of the fluorophore the photon can be absorbed, see Figure 1.2. If the photon has slightly more energy than between the electronic states it can still be absorbed and the excess energy brings the fluorophore in an even higher state of vibrational and/or rotational energy within \( S_1 \). Thus, a fluorophore can be excited by a range of wavelengths as long as the minimum energy is higher or equal to the \( S_0 - S_1 \) transition. The fluorophore is then relaxed very quickly to the lowest state of \( S_1 \) by internal conversion, phonons or some other process. From the ground state of \( S_1 \) the fluorophore then relaxes to one of the many states of the electronic ground state \( S_0 \), and transmits a range of wavelengths typically redshifted from the excitation wavelength. This is the fluorescence [2].

In confocal microscopy the contrast advantages of fluorescence is combined with a pinhole in the detection path that excludes a large portion of the light from planes out of focus [4]. The fluorophores in the sample are illuminated with a focused laser beam and in the confocal
image plane of the focus the pinhole is located, allowing only light originating from the focal plane to pass and hit the detector. The sample is scanned by either moving the sample or, more commonly, by a set of pivoting mirrors that deflects the laser beam in the $x$- and $y$-direction of the sample plane. Thanks to the pinhole the scanning can be repeated in several $z$-positions to obtain a set of slices to build a 3D-stack.

Confocal microscopy pushes the performance of light microscopy to the limit, but cannot break the diffraction limit.

### 1.2 Super resolution microscopy

Super resolution microscopy is a generic term for a number of techniques that in different ways can achieve resolution beyond the diffraction barrier. One can distinguish two types of far field super resolution techniques; wether they rely on a linear or a non-linear response of the fluorophores it’s illuminating.

Here a few of the most common techniques are briefly described together with a short summary of the BioAxial setup.

#### 1.2.1 SIM

Structured Illumination Microscopy or SIM illuminates the sample with sinusoidally modulated patterns of high frequency in various angles [5]. The modulation is generally done with a grating with variable orientation. The illumination pattern is overlaid with the unknown pattern in the sample which creates interference, so called Moiré fringes of lower frequency, see Figure 1.3(a).
With the knowledge of the spatial frequency of the illumination pattern and the measured frequency of the Moiré fringes, $\frac{1}{\Delta d}$, the spatial frequency of the sample can be calculated even if it is higher than the classic resolution limit. One can say that this procedure “moves” usually unresolvable frequencies of the sample into the observable region of the reciprocal space, $k$-space, and by doing so gives a 2-fold increase for the lateral resolution compared to the diffraction limit, $\frac{1}{\Delta x}$. Each illumination pattern can however only give additional information about high frequency structures of the sample in one direction, so the procedure has to be repeated in several angles to fully map the area, see Figure 1.3(b). A pair of two diametrically opposite red circles represent the extension of $k$-space that one illumination pattern of a certain direction provides. The illumination angle is rotated to cover more portions of $k$-space. One big advantage is that SIM is a wide field technique, which means fast acquisition. The technique exploits a pure physical phenomena so all types of fluorophores can be used.

![SIM pattern](image1)

![k-space](image2)

Figure 1.3: Structured illumination pattern creates Moiré fringes with the lateral pattern of the sample (a). With a sufficiently large number of illumination directions the area in $k$-space is doubled, corresponding to twice the normal resolution (b).

### 1.2.2 STED

STimulated Emission Depletion or (STED) is one of the nonlinear techniques. This technique uses the same type of spot illumination as confocal to excite the fluorophores, but immediately after, an additional beam shaped as a doughnut with a wave length matching the energy of the band-gap, stimulates emission from the fluorophores in an area around the PSF, see Figure 1.4. This leaves the fluorophores in the centre unaffected, and the PSF is shrunk [3], see Figure 1.5.

The doughnut shaped STED beam depletes all fluorescence in the vicinity of the excitation illumination, effectively shrinking the PSF. The diameter of the PSF, $\Delta r_{STED}$, is a non-linear function of the applied intensity of the STED beam, $I$, according to:

$$\Delta r_{STED} = \frac{\lambda}{2NA} \cdot \frac{1}{\sqrt{1 + \frac{I}{I_c}}}$$

1.2
where $I_s$ is the saturation intensity of the fluorophores. By sending an intensity high enough the size of the PSF can be made arbitrarily small [8].

### 1.2.3 PALM/STORM

Another way of achieving super resolution is by reducing the number of light emitting fluorophores to a very sparse distribution that has close to zero percent overlap. This is obtained by using so called photo-switchable fluorophores that initially are unsusceptible to the excitation light but can be made receptive by a light pulse of a certain wave length.

The two similar methods Photo Activation Localisation Microscopy (PALM) and Stochastic Optical Reconstruction Microscopy (STORM) are both stochastic ways to achieve super resolution by controlling the emission of light from the fluorophores [9,10]. The sample is first exposed by a wide field activating light pulse that, in a stochastic manner, makes a subset of the fluorophores susceptible for the subsequent exciting light pulse. The light from the sparsely distributed fluorophores is gathered in an image and the centroids of the single light emitters can be calculated with high precision. The fluorophores that once have been activated immediately bleaches out and are henceforth impossible to excite. The procedure is then repeated a number of times, typically a few thousand, to obtain a sufficient number of images and enough data to reconstruct a wide field image, see Figure 1.6.
1.3 Bioaxial super resolution

The Bioaxial system is a "black box" add-on feature for a standard confocal microscope. The idea is that the laser fibre you normally plug in to the confocal scanning head of the microscope is detoured through the Bioaxial system before entering the microscope. In the box some quite sophisticated beam shaping occurs.

The core of the system is a biaxial crystal. When light is transmitted along one of the (two) optical axes through a biaxial crystal a remarkable phenomenon called conical diffraction occurs. A focused beam is diffracted to a slant hollow cone within the crystal which is refracted into a hollow, perfectly circular, cylinder upon exiting the back faucet of the crystal. The conically diffracted light is composed of two components with different polarisation, which will be referred to as (the-) Fundamental and Vortex. By introducing polarising optics the two beam components can readily be separated or mixed in different proportions to obtain a family of various spatial distributions of the emerging beam.

The Bioaxial system has a typical polarimeter set up. This means that the biaxial crystal is placed in between polarising optics to be able to manipulate the polarisation of both the incident- as well as the transmitted light from the crystal. In the path before the crystal we have first a horizontal linear polariser and then a double pockels cell acting as polarisation generator (PSG). The two pockels cells are oriented with their fast axis in 45° and 0° respectively in this order in the optical path. This allows generation of any polarisation on the Poincaré sphere of the incident light. After the crystal another double pockels cell unit together with a vertical linear polariser acts as polarisation analyser (PSA). The two pockels cells are oriented as the PSG but in reverse, i.e. first 0° and then 45° in the optical path.

Currently Bioaxial use the so called half-moons in four different orientations together with the fundamental. The half-moons are oriented with the black line in 0°, 60°, 90° and 120° with the horizontal axis as reference.

![Figure 1.7: Polarimeter set up of the BioAxial system.](image)

![Figure 1.8: A simulation of the five different illumination distributions currently used with the BioAxial system.](image)
The sample is illuminated in a point scanning manner with the five distributions above. Each illumination pattern generates an image of the fluorescent response of the sample, which is analysed. Because of the different spatial features of each distribution each image contain independent information about the sample structure in a certain direction. One can say that the method has a lot in common with the SIM method, where the main difference is that while SIM is a wide field technique, BioAxial is a structured point illumination. The intensity information in the five different images is then run through a reconstruction algorithm which provides super resolution.

1.4 Vortices and super-oscillations

Singular optics and especially optical vortices has gained a lot of attention since a paper by Nye and Berry in 1974 [12]. The vortex is the most common example of an optical singularity. The vortex has a so called doughnut shape with zero intensity in the middle due to a phase singularity. The phase of the vortex has a helical structure and the number of $2\pi$ phase jumps along a fixed radius in the transverse plane of the beam is called the topological charge $l$, see Figure 1.9 and 1.10.

![Vortex and phase map](image)

(a) Vortex  (b) Phase map

Figure 1.9: A simulation of a vortex and the corresponding phase map in Fourier space. Scale bar in radians.

![Helical structure](image)

Figure 1.10: The helical structure of the phase of a $l = 1$ vortex. The image has been borrowed from [13].

Not only is the optical vortex an example of a optical singularity, it is also a so called super-oscillation [14,15]. A super-oscillation is a signal of any kind that locally oscillates arbitrarily fast for an arbitrarily long period of time, even though it is globally band limited. This means that the vortex can oscillate faster than the Nyqvist rate [16] [17], i.e., with a frequency that is faster than its fastest Fourier component [18].
A focused beam with a phase singularity, i.e. a vortex, has a super-oscillatory spatial frequency in the intensity profile. This means that the distance from one of the maxima to the singularity, in the intensity profile, is actually smaller than the FWHM of the airy disc and hence below the diffraction limit, see Figure 1.11.

If we look at the ratio
\[
\frac{\Delta x_{\text{fund}}}{\Delta x_{\text{vort}}} \approx 1.7
\] (1.3)
indicated in Figure 1.11, we see that the distance from maxima till minima of the fundamental is approximately 1.7 times larger than for the vortex.

The effect of super-oscillations and the consequence of an intensity profile with a spatial frequency below the diffraction limit is what is being utilised in the BioAxial system. The use of super-oscillatory optical effects has already been proposed for sub-diffraction imaging in various other ways before [15,19–22], but never in this simple and fast manner.
Chapter 2

Conical diffraction
The phenomenon of conical diffraction has been known for nearly two centuries. It was predicted 1832 by the Irish mathematician William Rowan Hamilton [23] and experimentally demonstrated by his colleague Humphrey Lloyd [24] just a few months later. It is possibly the first example in the history of science where mathematical theory led the way of experimental research and predicted a physical property. Conical diffraction appears in anisotropic crystals that have all three components of the dielectric factors different. This type of crystals are equipped with two optical axes, and are therefore called biaxial crystals. Conical diffraction has not received much attention throughout the years, and is only discussed in the most advanced optical textbooks. Recently, however, the phenomena has grown more and more popular and the number of publications on the topic has increased tremendously lately [25]. A notable reason for this lack of interest has been the difficulty to obtain large and pure enough crystals to be able to perform experiments. In more recent years the artificial growing techniques has been refined and the existence of crystals is no longer an issue. Another problem has been that the theory proposed by Hamilton, based on geometrical optics, has been too complicated to fully grasp for the majority. In the -70’s Belskii et. al could, by introducing par-axiality, put forward a full wave theory of the phenomenon [26,27] which gave a more thorough description. This was further simplified by Berry [28], who has done extensive research on the subject [29–31]. Peet [32,33] contributed comprehensive material about the mode converter capabilities a polarimeter setup with a thin biaxial crystal has. Manisipur [34] as well as Lunney et. al [35] has both provided non-technical approaches which are very enlightening.

The phenomenon manifests’ in two separate ways depending on along which path within the crystal the light travels. When an incident beam of light refracts into the crystal along one of the binormals, i.e. optical axes, a diverging cone of rays fans out within the crystal. The cone refracts into a hollow cylinder of light upon leaving the crystal, this is internal conical diffraction. When the incident light instead travels along one of the bi-radials, the light instead forms a diverging cone first after leaving the crystal, see Figure 2.1. This text will only be concerned with internal conical diffraction.

![Figure 2.1: Internal (a) and external (b) conical diffraction. The image can be found in its original in [35]](image-url)
2.1 Light propagation in crystals

First we will have a look at the propagation of light in anisotropic media, using the notation and terminology of [25] and [36].

For isotropic media the displacement vector $D$ is related to the electric vector $E$ by $D = \varepsilon E$, where $\varepsilon$ is the dielectric factor, a measure of the permittivity, for that particular medium. The dielectric factor is in turn related to the index of refraction, $n$, by $n = \sqrt{\varepsilon}$. The dielectric factor is generally frequency dependent, $\varepsilon(\omega)$, but the following discussion will be limited to monochromatic light.

The permittivity and thus the refractive index for an isotropic medium is described by a scalar and is therefore not direction dependent within this type of crystal. A propagating ray of light “sees” the same index of refraction regardless of propagation direction. For anisotropic media, however, the permittivity can no longer be described by a scalar, but rather a tensor. This has the consequence that the index of refraction is a function of propagation direction within the crystal.

$$D_i = \varepsilon_{ik}E_k = \begin{bmatrix} D_x \\ D_y \\ D_z \end{bmatrix} = \begin{bmatrix} \varepsilon_{xx}E_x & \varepsilon_{xy}E_y & \varepsilon_{xz}E_z \\ \varepsilon_{yx}E_x & \varepsilon_{yy}E_y & \varepsilon_{yz}E_z \\ \varepsilon_{zx}E_x & \varepsilon_{zy}E_y & \varepsilon_{zz}E_z \end{bmatrix}, \quad B = H$$

This means that the $D$ and $E$ no longer has to point in the same direction.

Looking to Maxwell’s equations for a monochromatic wave of frequency, $\omega$, we have:

$$i\omega H = c\nabla \times B, \quad i\omega D = -c\nabla \times H$$

For a plane wave propagating with wave vector $k$ in anisotropic media we can differentiate (2.2) to obtain:

$$\omega H/c = k \times E, \quad \omega D/c = -k \times H$$

where $c$ is the vacuum velocity of light. One can see that the three vectors $k$, $D$, and $H$ are mutually perpendicular. $H$ is perpendicular to $E$, which means that vectors $D$, $E$ and $k$ are coplanar. See Figure 2.2

![Figure 2.2: Relative vector positions for anisotropic media. Unlike isotropic media, $E$ is no longer parallel to $D$, and hence $S$ is no longer parallel to $k$. The image can be found in its original in [25].](image)
The Poynting vector, \( \mathbf{S} \), defined as \( \mathbf{S} = \mathbf{E} \times \mathbf{H} \), which is the direction of rays and also gives the direction of energy flux, is also shown. The vector \( \mathbf{S} \) is coplanar with \( \mathbf{E} \), \( \mathbf{D} \), and \( \mathbf{k} \), and the angle, \( \alpha \), between \( \mathbf{E} \) and \( \mathbf{D} \) is the same as between \( \mathbf{k} \) and \( \mathbf{S} \). With the wave vector \( \mathbf{k} \) we can now define a vector \( \mathbf{n} \), which describes the magnitude of the refractive index as a function of direction.

\[
\mathbf{k} = \frac{\omega \mathbf{n}}{c} \tag{2.4}
\]

The differentiation of Maxwell’s equations, eq. (2.3) can then be rewritten with eq. (2.4) as:

\[
\mathbf{H} = \mathbf{n} \times \mathbf{E}, \quad \mathbf{D} = -\mathbf{n} \times \mathbf{H} \tag{2.5}
\]

and by substituting the first equation (2.5) into the second, we have:

\[
\mathbf{D} = \mathbf{n} \times (\mathbf{E} \times \mathbf{n}) = n^2 \mathbf{E} - (\mathbf{n} \cdot \mathbf{E}) \mathbf{n} \tag{2.6}
\]

By equating the components of eq. (2.6) in accordance with eq. (2.1) we obtain three linear homogeneous equations for the three components, \( k = x, y, z \), of \( \mathbf{E} \)

\[
n^2 E_i - n_i n_k E_k = \varepsilon_{ik} E_k \tag{2.7}
\]

Equation (2.8) is called Fresnel’s equation and is a fundamental equation of crystal optics. Generally eq. (2.8) determines the dispersion relation, i.e. the frequency as a function of wave vector. Given that we presently only are dealing with monochromatic light, \( \omega \), and thus the dielectric factors \( \varepsilon \) are here constants. Eq. (2.8) then gives the magnitude of the wave vector as a function of direction. The solution to eq. (2.8) is quadratic in \( n^2 \) so for, generally, any given direction \( \mathbf{n} \) there exists two solutions. Mapping all possible directions gives a two sheeted surface known as the wave surface, describing the generated wave vectors as a function of direction. A uniaxial crystal has two of the principal axes of the dielectric tensor \( \varepsilon_{ik} \) equal. If we take \( x \) and \( y \) to be equal and put \( \varepsilon_x = \varepsilon_y = \varepsilon_\parallel \), and \( \varepsilon_z = \varepsilon_\perp \) in eq. (2.8) the left hand side is a product of two quadratic factors

\[
n^2(\varepsilon_x n_x^2 + \varepsilon_y n_y^2 + \varepsilon_z n_z^2) - [n_x^2 \varepsilon_x (\varepsilon_y + \varepsilon_z) + n_y^2 \varepsilon_y (\varepsilon_x + \varepsilon_z) + n_z^2 \varepsilon_z (\varepsilon_x + \varepsilon_y)] + \varepsilon_x \varepsilon_y \varepsilon_z = 0 \tag{2.8}
\]

Equation (2.8) is called Fresnel’s equation and is a fundamental equation of crystal optics. Generally eq. (2.8) determines the dispersion relation, i.e. the frequency as a function of wave vector. Given that we presently only are dealing with monochromatic light, \( \omega \), and thus the dielectric factors \( \varepsilon \) are here constants. Eq. (2.8) then gives the magnitude of the wave vector as a function of direction. The solution to eq. (2.8) is quadratic in \( n^2 \) so for, generally, any given direction \( \mathbf{n} \) there exists two solutions. Mapping all possible directions gives a two sheeted surface known as the wave surface, describing the generated wave vectors as a function of direction. A uniaxial crystal has two of the principal axes of the dielectric tensor equal. If we take \( x \) and \( y \) to be equal and put \( \varepsilon_x = \varepsilon_y = \varepsilon_\parallel \), and \( \varepsilon_z = \varepsilon_\perp \) in eq. (2.8) the left hand side is a product of two quadratic factors

\[
(n^2 - \varepsilon_\parallel)[\varepsilon_\parallel n_x^2 + \varepsilon_\perp (n_x^2 + n_y^2) - \varepsilon_\perp \varepsilon_\parallel] = 0 \tag{2.9}
\]

The roots to eq. 2.9 is two quadratic equations

\[
n^2 = \varepsilon_\perp \tag{2.10a}
\]

\[
\frac{n_x^2}{\varepsilon_\perp} + \frac{n_y^2 + n_z^2}{\varepsilon_\parallel} = 1 \tag{2.10b}
\]

We can see that eq. (2.10a) describes a sphere and that eq. (2.10b) describes an ellipsoid. These are the two expressions that generates the double sheeted wave surface. In figure 2.3 an octant of the wave surface is displayed. The surfaces touch on the z-axis, in this direction the two indices of refraction is evidently equal. A line through this point and the origin is known
2.1. LIGHT PROPAGATION IN CRYSTALS  \hspace{1cm} \text{CHAPTER 2. CONICAL DIFFRACTION}

Figure 2.3: An octant of the wave surface of a uniaxial crystal. The image has been slightly altered to fit the notation of this text and can be found in its original in [37].

as the optical axis of the crystal.

In a biaxial crystal all three principal axes of the dielectric tensor are different. If we assume:

$$\varepsilon_x < \varepsilon_y < \varepsilon_z \quad (2.11)$$

and by putting $n_z = 0$ in eq. (2.8) yields:

$$(n^2 - \varepsilon_z)(\varepsilon_x n_x^2 + \varepsilon_y n_y^2 - \varepsilon_x \varepsilon_y) = 0 \quad (2.12)$$

The roots to this equation gives the sections in the $xy$-plane, which are:

$$n^2 = \varepsilon_z \quad (2.13a)$$

$$\frac{n_x^2}{\varepsilon_x} + \frac{n_y^2}{\varepsilon_y} = 1 \quad (2.13b)$$

Evidently, equations (2.13) describes a circle and an ellipse in the $xy$-plane, see Figure 2.4b. With a similar approach, i.e. putting $n_x = 0$ and then $n_y = 0$, we find that the sections in the $yz$- and $xz$-plane, respectively, also consists of a circle and an ellipse. With condition (2.11) we can see that in the $yz$-plane the ellipse lies outside the circle and in the $xz$-plane they intersect in four points, see Figure 2.4a and 2.4c, respectively.
Figure 2.4: The wave surfaces for a biaxial crystal in three planes. In (a) the $yz$-plane is shown, in (b) the $xy$-plane is shown and in (c) the $xz$-plane is shown. The images can be found in their original in [25].

In figure 2.5 the corresponding octant of the wave surfaces is displayed. The wave surfaces can be seen to intersect in one point here at an angle $\beta$ to the $z$-axis rather than coinciding with it like for a uniaxial material.

Figure 2.5: An octant of the wave surface of a biaxial crystal. The two surfaces intersect in the $xz$-plane at an angle $\beta$ to the $z$-axis. Through this singularity and the origin runs one of the optical axes. The image has been slightly altered to fit the notation of this text and can be found in its original in [36].
2.1. LIGHT PROPAGATION IN CRYSTALS  

CHAPTER 2. CONICAL DIFFRACTION

As can be seen in Figure 2.6 the two surfaces actually intersects in four points, one in each quadrant of the \( xz \)-plane. In these points all three indices of refraction coincide, the wave surface is singular. The two lines that can be drawn through two opposing singularities through the origin are the optical axes. The direction in which the interception will occur is obtained by differentiating eq. (2.8). We obtain

\[
\begin{align*}
    n_x [\varepsilon_y (\varepsilon_y + \varepsilon_z) - \varepsilon_x n_x^2 - (\varepsilon_x n_x^2 + \varepsilon_y n_y^2 + \varepsilon_z n_z^2)] &= 0 \\
    n_y [\varepsilon_y (\varepsilon_x + \varepsilon_y) - \varepsilon_y n_x^2 - (\varepsilon_x n_x^2 + \varepsilon_y n_y^2 + \varepsilon_z n_z^2)] &= 0 \\
    n_z [\varepsilon_z (\varepsilon_x + \varepsilon_y) - \varepsilon_z n_x^2 - (\varepsilon_x n_x^2 + \varepsilon_y n_y^2 + \varepsilon_z n_z^2)] &= 0
\end{align*}
\]

(2.14)

We know that the interception lies in the \( xz \)-plane, so by putting \( n_y = 0 \) the two remaining equations gives

\[
\begin{align*}
    n_x^2 &= \frac{\varepsilon_z (\varepsilon_y - \varepsilon_x)}{\varepsilon_z - \varepsilon_x} \\
    n_z^2 &= \frac{\varepsilon_x (\varepsilon_z - \varepsilon_y)}{\varepsilon_z - \varepsilon_x}
\end{align*}
\]

(2.15)

The angle these vectors make with the \( z \)-axis is \( \beta \) according to

\[
\frac{n_x}{n_z} = \tan \beta = \pm \sqrt{\frac{\varepsilon_x (\varepsilon_y - \varepsilon_x)}{\varepsilon_x (\varepsilon_z - \varepsilon_y)}}
\]

(2.16)

With the wave surface it is possible to construct a reciprocal surface known as the ray surface. The ray surface defines the direction of propagation of rays in the corresponding direction of the wave surface. The normals of the tangent planes of the two solutions of the wave surface gives the corresponding ray direction. I.e. in a given direction the wave surface has two solutions. The normal to the tangent plane of each of these surfaces gives the direction of two separate rays, known as the ordinary- and the extraordinary ray. This is also known as the phenomenon of birefringence, or double refraction. This is valid for any \( \mathbf{k} \), except when \( \mathbf{k} \) coincides with the optical axis. At a point along the optical axis the two surfaces intersect and the two wave surfaces has the form of two shallow cones that meet in a diabolic point, an expression coined by Berry [31], see Figure 2.7.
Hamilton realised that the normals to the wave surface in the diabolical point are not defined, there exist an infinite number of normals. If we choose $\mathbf{k}$ along the optic axis of the crystal any perpendicular direction of $\mathbf{D}$ is allowed and when rotating around $\mathbf{k}$ the $\mathbf{E}$ vector and hence $\mathbf{S}$ describes a cone. This means that the incident wave fans out in an infinite number of rays that forms the surface of the cone with semi angle $A$, depicted in Figure 2.7.

Figure 2.7: The two wave surfaces intersects in a diabolical point on the optical axis. When rotating $\mathbf{D}$ around $\mathbf{k}$, $\mathbf{S}$ fans out the ray cone with semi angle $A$. The image has been slightly altered to fit the notation of this text and can be found in its original in [31]

With the assumption made earlier in eq. (2.11), the angle $A$ is given by:

$$A = \frac{1}{n_y} \sqrt{(n_y - n_x)(n_z - n_y)} \quad (2.17)$$
2.1. LIGHT PROPAGATION IN CRYSTALS  CHAPTER 2. CONICAL DIFFRACTION

Considering a circularly symmetric Gaussian beam with a circular polarisation of either handedness:

\[ E_0(r) = e^{-\frac{r^2}{2w^2}} \]  

(2.18)

with wave number \( k_0 \) and beam waist \( w \) incident on a polished surface perpendicular to one of the optical axes of a biaxial crystal, see Figure 2.8. The emerging beam depends on the following variables:

\[ \rho_0 \equiv \frac{R_0}{w} = \frac{Al}{w}, \quad \rho \equiv \frac{r}{w}, \quad \zeta \equiv \frac{l + (z - l)n_y}{n_y k_0 w^2} \]  

(2.19)

where \( w \) is the beam waist width. \( r \) and \( z \) are cylindrical polar coordinates, with \( z \) measured from the waist of the beam and \( r \) measured from the centre axis of the emerging cylinder of light. The radius, \( R_0 \), of the light cylinder depends on the angle \( A \) of the cone within the crystal, i.e. the composition of refractive indices. Moreover it depends on the thickness, \( l \), of the crystal: \( R_0 = Al \). \( \rho \) and \( \zeta \) denotes the radial position and propagation distance in units of beam waist, \( w \) and Rayleigh range, \( k_0 w^2 \), respectively. \( \zeta \) is measured from \( z = l(1 - 1/n_y) \) which is known as the focal image plane (FIP), the plane of the virtual image of the entrance facet of the crystal for an equivalent isotropic crystal with index \( n_y \).

For the effects of internal conical diffraction to appear distinctively the condition \( \rho_0 \gg 1 \rightarrow R_0 >> w_0 \), i.e. a thick crystal, has to be fulfilled. Otherwise the fine details will be obscured by the beam width.

In Figure 2.9 the effect of a biaxial crystal on an incident beam can be seen to go from birefringence to internal conical diffraction as the crystal is stepwise turned towards the optical axis.

Figure 2.8: Internal conical diffraction of a focused Gaussian beam incident along one of the optical axes of a biaxial crystal. The image can be found in its original in [38]

\[ \rho_0 \equiv \frac{R_0}{w} = \frac{Al}{w}, \quad \rho \equiv \frac{r}{w}, \quad \zeta \equiv \frac{l + (z - l)n_y}{n_y k_0 w^2} \]  

Figure 2.9: As the crystal is rotated and the beam approaches the optical axis we can see how the light goes from being doubly refracted in (a) to complete conical refraction in (c). The image can be found in its original in [25]
With a circular polarised input beam the polarisation profile of the ring undergoes a $\pi$ rotation to the opposing point along the circumference around the axis. Each point on the ring is linearly polarised and two diametrically opposite points has orthogonal polarisation, see Figure 2.10.

![Figure 2.10](image_url)

Figure 2.10: The polarisation around the ring of conical diffraction. The image can be found in its original in [25]
2.2 Wave theory

A derivation of the wave theory is beyond the scope of this text and only the final equations will be presented. Notation of Berry [28] and O’Dwyer [25] has been adopted.

The exact paraxial solution to the $E$-field of the conically diffracted light is given below:

\[
E(R, z) = B_0(R, R_0, Z) \begin{bmatrix} e_x \\ e_y \end{bmatrix} + B_1(R, R_0, Z) \mathbf{M}(\theta_p) \begin{bmatrix} e_x \\ e_y \end{bmatrix}
\] (2.20)

\[
B_0(R, R_0, Z) = k \int_{0}^{\infty} P \cos(kPR_0)a(P)J_0(kPR)e^{-\frac{i\mu P^2 Z}{2}}dP
\] (2.21)

\[
B_1(R, R_0, Z) = k \int_{0}^{\infty} P \sin(kPR_0)a(P)J_1(kPR)e^{-\frac{i\mu P^2 Z}{2}}dP
\] (2.22)

where $J_0$ and $J_1$ are Bessel functions of 0:th and first order respectively. $k = n_y k_0$ is the crystal wavenumber along the optical axis which enables the radial position $R = (x, y)$ to be described around the azimuthal angle, $\theta$, of the cone in terms of transverse wave vectors $kP = k(P_x, P_y) = kP(\cos \theta_p, \sin \theta_p)$. $P << 1$ because of par-axiality. $\begin{bmatrix} e_x \\ e_y \end{bmatrix}$ is the polarisation Jones matrix of the incident light, see Appendix A.

\[
\mathbf{M}(\theta_p) = \begin{pmatrix} \cos \theta_p & \sin \theta_p \\ \sin \theta_p & -\cos \theta_p \end{pmatrix}
\]

$a(P)$ is the Fourier transform of the incident electric field, and $Z = l + (z - l)n_y$ is the distance from the FIP measured in units of $n_y$, with the remaining notations given above.

Thus, the emerging field is built up of two Bessel components which we will refer to as the Fundamental and the Vortex beam, see Figure 2.11.

![Figure 2.11: The two field components building up the electric field after the crystal.](image)

Looking at eq. (2.20) one can see that the polarisation of the $B_0$-component is preserved while the matrix $\mathbf{M}(\theta_p)$ converts the polarisation of the $B_1$-component to be orthogonal to the former.

As mentioned before in, Section 1.3, the two components can hence be separated and/or mixed in different proportions to obtain a variety of spatial intensity profiles by introducing polarisation optics in the optical path.

Hamilton’s theory only considered perfectly parallel rays to the optical axis. Experimentally, however, some of the rays always have a small deflection angle to the optical axis. These rays
will hence not be conically refracted but will instead undergo double refraction. Consequently the emerging cylinder of light from the back facet of the crystal does not only consist of one ring but two. The two rings are separated by the so called Poggendorff dark ring after the man who first made the observation [39].

![Figure 2.12: Experimental image of the FIP of a conically diffracted beam. Z = 0 and ρ₀ = 42.1. The image can be found in its original in [25].](image)

2.3 Polarimeter set up

In BioAxial one realised that not only thick crystals are of interest. With \( w_0 \sim R_0 \), i.e. a thin crystal, the main features of the conical diffraction phenomenon is preserved in terms of amplitude, phase and polarisation. One realised that with the condition \( w_0 \sim R_0 \) the spatial pattern, actually, simplifies significantly and that a thin crystal can be used to transform the Point Spread Function (PSF) of the an incident beam. This is achieved by enclosing the biaxial crystal in a polarimeter set up where one can obtain a large variety of distributions depending on the incoming and emerging polarisation states in the polarimeter. This simple optical set-up has the ability to switch from one pattern to another pattern with another topology in a time of microseconds - or below. Moreover, these patterns are perfectly co-localized, being produced by the same primary optical beam. This has also been shown by Peet [32,33,40].

When circularly polarized light is incident on the crystal the outgoing beam \( B_0 \) emerges with the same polarization, while the second component \( B_1 \) is also circularly polarized, but with the opposite handedness to the input beam. This means that the two components can easily be separated with a circular analyzer of either handedness.

By manipulating the state of polarization in the PSG and PSA a range of distributions can be generated, see Figure 2.13. When the input light is linearly polarized the outgoing beam acquires an azimuthal intensity modulation. When the PSA is linear and orthogonal to the input the the two field components are allowed to interfere and the outgoing beam profile consists of both \( B_0 \) and \( B_1 \), and the previously mentioned “half-moon”-pattern is obtained. All of these distributions can also be rotated around their beam axis to obtain e.g. the four “half-moon”-patterns presented in Figure 1.8.
Figure 2.13: The family of spatial distributions that can be generated with a biaxial crystal and polarising optics. The upper horizontal axis indicates the polarisation state of the PSG and the vertical axis the state of the PSA.
Chapter 3

BioAxial super resolution
The BioAxial system module is, as mentioned before, essentially a black box, through which the laser is fed before entering a standard confocal microscope. The optical components of the module is mounted on a small bread board, see photo in Figure 3.1. The optical path contains two mirrors to make the module more compact.

![Figure 3.1: Photograph of the bread board set up of the BioAxial system. The C2 scanning head of the Nikon microscope is visible in the upper left corner.](image)

The main drawbacks are a very limited field of view (FOV), a long acquisition time and a long reconstruction time.

The optical hardware gives a resolution improvement of approximately a factor 2 through the super-oscillation pattern. Together with a stochastic reconstruction algorithm the final resolution improvement is somewhere between a factor 2-4. The exact figure is not known, and it also depends a lot on the structure of the sample.

### 3.1 System set up

The system has two laser inputs. One of them is called the bypass path. The bypass path allows usage of the microscope just like an ordinary confocal microscope, description follows. The other is the BioAxial beam shaping path, which will be described in detail below. See Figure 3.2 for reference.

The laser fibre enters a collimator and the light is then reflected 90° and horizontally polarized in the first polarizing beam splitter (PBS 1). The light then enters a double pockels cell. The first of the two pockels cells is oriented with its fast axis in the 45°-direction, and the second in the 0°-direction. These two components, i.e. PBS 1 and the double pockels cell constitute the polarization state generator (PSG). With this set-up any polarization state on the Poincaré sphere can be generated. From the PSG the light is focused with a $f = 90$ mm doublet lens and reflected with a mirror, oriented 45° to the optical axis, into the biaxial crystal. After passage
through the crystal the beam is then reflected with another mirror at 45° and re-collimated with an identical doublet lens. The light then propagates into the polarization state analyzer (PSA), consisting of a double pockels cell and a vertically polarizing beam splitter (PBS 2). The pockels cells are oriented with their fast axis in the 0°- and 45° direction respectively along the optical path.

Everything emerging from the PSA is vertically polarized and this is not optimal for every distribution. To be able to control the polarization of the light that enters the microscope an additional pockels cell is located just after the PSA. The last pockels is oriented with its fast axis in the 45° direction and can reach polarization states along a meridian around the Poincaré sphere. The half moon distributions works best with linear polarization but the fundamental and the vortex works best when circularly polarized. The polarization of the excitation light also becomes important when using high numerical aperture objectives because of vectorial effects no longer are negligible. The fifth pockels cell is followed by a coupling lens that focuses the light into the entrance port of the scanner head of the microscope. This is done to imitate the behaviour of a point source, i.e. a fibre, which is the normal way of plugging the laser to the microscope. This means that the whole BioAxial module has to be very well aligned with the actual microscope itself. For that and other reasons, like vibration reduction, both the microscope and the BioAxial module is mounted and aligned on an pneumatic optical table. For future versions the output of the BioAxial module is planned to be fibered to allow for a complete stand-alone system that is easily plugged to the microscope. Since the system is very robust with no moving parts at all, there is really no need for it to be on an optical table. In addition a high voltage power supply is coupled to each of the pockels cells.

In the bypass path the laser goes straight to PBS 2 where it is reflected 90°. From there the bypass shares the remaining path with the BioAxial path through the fifth pockels cell and the coupling lens before entering the scanning head. The laser beam in this path is not spatially altered like in the other. The only thing that can change is the polarization.

![Figure 3.2: Schematic illustration of the optical path in the BioAxial system.](image)
The current version of the BioAxial module is mounted on a Nikon Eclipse Ti inverted microscope. The microscope employs the stratum structure and has two levels in the optical path where light can be extracted to allow for two back-ports with accompanying filter turrets. On the top level back-port a Hamamatsu EMCCD camera is mounted. The EMCCD camera acquires the fluorescent light from the sample during BioAxial measurements. On the bottom level an Epix camera is mounted. This camera will be used to track the laser position during scanning, this will be discussed in more detail in Section 4.3. In addition a photomultiplier tube (PMT) is coupled to the C2 scanning head which is used to acquire confocal data. In the filter turrets cubes with dichroic mirrors and filters are used to send the light in the right direction depending on which imaging modality is used. When standard confocal imaging is done, no filter at all is used since the fluorescent light from the sample should go back the same way it came from in order to be acquired by the PMT in the scanning head. When BioAxial measuring is done a cube is placed in the second filter turret level. The cube transmits the 488 nm excitation light to the sample but reflects the ≈520 nm fluorescent light on the camera.

The microscope is equipped with Nikons Perfect Focus System (PFS), which makes sure that the focus is kept at a certain level at all times without drifting. The PSF works with an infrared beam of light that is reflected on the sample surface and continuously monitored with an offset lens and a line-CCD. If e.g. vibrations or thermal drift is introduced the PFS system measures the offset and compensates by refocusing the objective.

Three different objectives are used for BioAxial measurements, a PlanApo 40x 0.95 NA air, a PlanApo 60x 1.2 NA water immersion and a PlanApo 100x 1.49 NA oil immersion objective. The laser used is a 488 nm Oxxius Laserboxx diode laser with an maximum output power of 50 mW.

3.2 Super resolution scheme

The BioAxial system module works essentially as a mode converter. On the one end you input a Gaussian beam and from the other you collect a completely different spatial distribution, depending on what voltages you put on the pockels cells. The current set-up employs one biaxial crystal and can generate a family of spatial light distributions, as explained above, see
3.2. SUPER RESOLUTION SCHEME  CHAPTER 3. BIOAXIAL SUPER RESOLUTION

figure 2.13. In addition to these distributions, each distribution can be azimuthally rotated by adjusting the PSG and PSA.

The increase in resolution is a result of two factors, optical hardware and mathematical treatment in the reconstruction algorithm. On the hardware side the resolution increase comes from the fact that the illumination pattern, which for the moment is the “half-moon”, see Figure 1.8, is a degeneration of the vortex which itself is an example of a super-oscillation. The distance from one of the two maxima to the zero is approximately 2 times smaller than the FWHM of the fundamental. The half moon patterns has its black line oriented in four different directions. Each one of the four orientations of the distributions gives sub-diffraction information in that particular direction. The method has its similarities with SIM although this is structured point illumination not wide field. Each of the half moon patterns moves information from an approximately 2 times larger $k$-space into the observable region, corresponding to the direction the black line is oriented. The four patterns together maps out a good coverage of the $k$-space, see Figure 3.4.

![Figure 3.4](image)

Figure 3.4: The available area in $k$-space, and thus lateral frequency range in the sample, is increased.
Chapter 4
Development
There are a number of development directions that is investigated simultaneously in BioAxial. To be able to make the BioAxial module a competitive product for the super resolution market there are several prerequisites demanded by the consumers that has to be fulfilled.

- It has to be independent of the objective used, i.e. all magnifications and numerical apertures needs to be compatible with the technique. This mainly applies to high numerical aperture and high magnification objectives, since it is these that is most desirable to use with super resolution. It is also because these objectives that offers the most resistance and complexity to be integrated in any optical system, mainly due to the vectorial effects of the electric field.

- It has to offer a reasonably large field of view (FOV). Closely linked to the FOV is the acquisition time. A very fast acquisition time is not only convenient but an absolute necessity to be able to record fast occurring events like live moving cells. The FOV and the acquisition time counteract each other and a tradeoff between the two is necessary.

- Also the calculation time for the reconstruction has to be reasonable for the system to be convenient and user friendly.

- Good spatial resolution is of course of interest, but one should not forget about axial resolution. The axial resolution is inherently worse than the spatial because the PSF is always elongated in the $z$-direction compared to $x,y$-direction [7]. The diffraction limit in the $z$-direction is approximately two times larger than in the $x,y$-direction. The most advantageous would be to have isotropic resolution.

### 4.1 Simulation of distributions

To be able to accurately simulate the distributions in the BioAxial system a graphical user interface (GUI) called Distribution Simulation was built on the Matlab platform, proceeding from the equations (2.20-2.22) set up in Section 2.2. The GUI allows the user to simulate the focused outcome of a Gaussian input beam depending on the polarization state of the PSG and the PSA. The beam is simulated in a user defined range around the focus in units of Rayleigh range. The user can also choose-, between three different biaxial crystals, the thickness of the crystal, the wave length of the light and the NA of the focusing optics.

The PSG and PSA has three settings: Linear, Circular and Elliptical polarization. For the linear and elliptical settings the orientation can be set and in addition the ellipticity of the elliptical can be chosen. For the circular polariser the handedness of the polarisation can be set.
4.2 High numerical aperture objectives

With a high numerical aperture objective, such as 1.49, vectorial effects of the electric field of the light become more and more prominent. A 1.49 NA objective has a very short working distance together with a very large angle under which it collects and transmits light. This means...
that the $E$-field, i.e., the polarization will have a non-negligible component in the $z$-direction of the light path. The BioAxial system relies to a high extent on the polarization in different areas of the beam profile of the excitation light, and this effect could have a significant effect on the distributions.

With the 1.2 NA objective, however, the vectorial effect seems to be of minor importance, much less than what we expected. As can be seen below, the result of a bead calibration with 100 nm beads, the shape of the distributions is very much like the desired. A measurement of the contrast and lobe distance also shows good result.

For the 1.49 NA objective the shape of the distributions suffers a lot due to the vectorial effects. The effects seems to blur the distributions. A significant amount of energy is also transferred to a type of side lobes in various directions depending on the distribution. This can be a problem of solely vectorial effects, but it is likely to be a combination of vectorial effects and a small tilt somewhere in the optical path. The idea of the fifth pockels cell is to optimize the polarization for each distribution to minimize the vectorial effects when using the high NA objectives. Unfortunately the effect on the distributions of the fifth pockels cell is only a rotation of the side lobes, which indicates that the problem is presumably due to a tilt of one or several of the optical components in the path.

4.3 Fast scan

As mentioned before the present BioAxial system is measuring in a pretty slow point scanning manner. When measuring with a 40x 0.95 NA air objective the size of the ROI is normally chosen to $2 \times 2 \, \mu m$ or $3 \times 3 \, \mu m$ with a step size of 100 nm. When using a 60x 1.2 NA air or a 100x 1.49 NA objective the step size is chosen to 60 nm and 40 nm respectively. The ROI, however, has the same number of pixels so the FOV scales down accordingly. These step sizes fulfil the Nyqvist criterion and has been chosen to optimize the sample densities required for the reconstruction algorithm. Depending on the brightness of the sample the time to acquire each frame varies. With a very bright sample the exposure- and light gathering time can be as low as 2 s for each stimulation point and with lesser bright samples it can exceed 3 s. It is the user that choses the time ratios for the illumination. This means that a typical measurement of a $2 \times 2 \, \mu m$ ROI takes about 20 min and a $3 \times 3 \, \mu m$ takes up to 1 h. A very long process indeed, and the ability to measure live cells with this set up is of course non-existent.

Therefore one is currently developing an extension of the first prototype called the Fast scan. The new version requires some additional hardware such as a second camera and an additional cube in the first filter turret of the microscope. It also requires an update of the reconstructing algorithms to be able to deal with a new type of raw data.

The goal of the fast scan is of course to be able to run measurements in much faster way. This is done by measuring in a sweeping line scan with one of the distributions at a time, rather than point scanning. Each line has to be swept five times, one for each distribution. But this means that the position of the excitation beam on the sample at any given time is not known, as it is when point scanning. Therefore a filter is inserted in one of the levels in the microscope stratum structure. The filter in the turret reflects about 10% of the incoming light on the second camera before it enters the objective. The second camera tracks the exact position of the laser beam in realtime.
The first experimental results of the Fast scan are very promising and points to a future where the acquisition time will be reduced by several factors.
Chapter 5

Measurements
The performance of the BioAxial system has been evaluated by correlative measurements. A large variety of sample structures has been imaged with the three modalities: BioAxial Super Resolution (BSR) mounted on a Nikon Eclipse C2 inverted confocal microscope; Confocal Laser Scanning Microscope (LSM) with the same microscope and finally Zeiss Elyra 3D Structural Illumination Microscopy (SIM). The results of, chiefly, BSR and SIM is compared. SIM is regarded as the ground truth. In the following the description of the objective in use for each measurement will be on the form: \(<\text{magnification}\> \times \,<\text{numerical aperture}\,> \,\text{NA }<\text{immersion medium}>\).

To be able to accurately reconstruct the measurement done with BSR the system needs to be calibrated. Essentially the reconstruction algorithm needs to know the shape of the different distributions and the positioning of the illumination spots. This is done by the means of so called Bead- and Scanner calibration. Bead- and Scanner calibration has to be done with the same voltages for the distributions of the pockels cells as the measurement itself. This is very important since the voltages determines the shape of the distributions. The calibration can be done either before or after the measurement, but has to be done within reasonable time of the measurement, typically a few days either before or after the measurement.

5.1 Calibration

In order for the system to work properly it needs to be calibrated. This is done with two separate calibration procedures: Scanner calibration and Bead calibration.

5.1.1 Scanner calibration

The scanner calibration is done to provide information about the coordinates of the spot illumination on the sample in respect to the coordinate of the acquired fluorescent light spot on the camera. This enables the reconstruction algorithm to accurately determine the exact position in the sample of an arbitrary spot acquired on the camera. It also eliminates the potential problem of a misaligned camera. The scanning illumination is running in a horizontal-vertical direction and if the camera where to be mounted with an angle in respect to the ROI of the sample, the resulting image would also be misaligned. The angle is calculated from the scanner calibration and compensated for.

The calibration is done with the fundamental distribution on an uniform sample. The sample is a standard microscope glass slide with a cover glass that has been marked with a regular highlighting pen.

The sample is illuminated with a number of points in a grid pattern, see Figure 5.1. The position of every illumination point in the microscope software (NIS) is known and then compared with the calculated centroid of each point in the acquired image.

5.1.2 Bead calibration

A bead calibration is primarily done to map the shape of the spatial profile of the distributions that is used to illuminate the sample. For the moment the BioAxial system use the so called half-moons in four different orientations together with the fundamental distribution. It is very important to have a good contrast between the two lobes of the half-moons. Ideally the
intensity should be zero along the line in between the half-moons. Another important parameter is the distance between the two lobes of the distribution. The distance should not exceed the size of the Airy disc. In practice the distance between the peaks of the two lobes is measured.

The sample used for this calibration is, of course, beads. More precisely synthetic fluorescent beads of varying size depending on the magnification of the objective used.

For the 40x 0.95 NA air objective the bead calibration is normally done by scanning a ROI with a size of $2 \times 2 \ \mu m^2$ of the sample with a step size of 100 nm. A smaller step size of 50 nm has been tried with some quality improvements, but the acquisition time is however too long (> 1 h).

As explained in 3 each point is illuminated with the five distributions and five different images are acquired, one each of the fluorescent light that each of the distributions give rise to, with the camera. Each of these sub images is then translated and superimposed on top of each other to form the so called de-scan-image, see Figure 5.2.

Figure 5.1: Scanner calibration done with 40x 0.95 NA air. The distance between dots are 5 µm.

Figure 5.2: Bead calibration of 100 nm beads done with 40x 0.95 NA air objective.
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The de-scan image is then analysed with a Matlab GUI developed in BioAxial, called Quickview. Quickview allows analyzation of the distance between the lobes and the contrast between the peak value of the lobes and the dark line in between the lobes.

With the Bioaxial system three objectives are in use: 40x 0.95 NA air; 60x 1.2 NA water and a 100x 1.49 NA oil. With the 40x objective, 100 nm beads has been found to be sufficiently small. For the two other objectives the preferable size of the beads is smaller, perhaps 40 nm. These beads are however very hard find of sufficient quality. The distribution of beads on the sample needs to be sparse enough to be able to calibrate on a single bead. Because of their size they are also quite weakly fluorescent. So far Bead calibration for the the other two objectives has only been successful with 100 nm beads. The main drawback with using too large beads with higher magnification is that the contrast between the lobes is inherently worse.

Below calibrations and cross sections done with 60x 1.2 NA water and 100x 1.49 NA oil objectives is presented. Note the the decreasing distance between the lobes as the NA is increasing.

Figure 5.3: Cross sections of the distributions if Figure 5.2

![Cross sections of the distributions](image1)

![Cross sections of the distributions](image2)

Figure 5.4: Bead calibration of 100 nm beads done with 60x 1.2 NA water objective.
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Figure 5.5: Cross section of the distributions in Figure 5.4.

Figure 5.6: Bead calibration of 100 nm beads done with 100x 1.49 NA oil objective.
5.2 Fixed biological samples

The experimental protocol for correlative measurements is as follows. All measurements are done with the three modalities described above: BSR; SIM and LSM. The LSM image is normally done with the same objective as the BSR and always with a step size at the Nyquist criterion for diffraction limit.

To be able to find the same structure of the measured area, the sample is marked on its backside with the small dot of a waterproof marker pen. A confocal image is also acquired with the widest field of view and is used as a map to find the same area with the SIM.

5.2.1 Microtubules

Microtubules is one of three essential components of the cytoskeleton, the structural framework of cells. Microtubules are hollow tubes of approximately 25 nm in diameter and builds up a big network of tubes running in all directions within the cell. The microtubules determine the shape of the cell and are also responsible for some cell movements [41]. Here U373 Glioma cell microtubules stained with Alexa 488 has been measured with three different modalities, see figure 5.8. In pink we have BSR done with 60x 1.2 NA water, in white Nikon Confocal with the same objective and in blue Zeiss Elyra 3D SIM done with 63x 1.4 NA oil.
5.2. FIXED BIOLOGICAL SAMPLES

CHAPTER 5. MEASUREMENTS

(a) Cross section 1

(b) Cross section 2

Figure 5.9: Cross sections from Figure 5.8. Subfigure (a) correspond to line 1 and (b) to line 2.

Figure 5.8: Microtubules. BioAxial in pink with 60x 1.2NA water and in white Nikon Confocal with the same objective. Zeiss Elyra 3D SIM in blue with 63x 1.4NA oil.
5.2. FIXED BIOLOGICAL SAMPLES

5.2.2 Actin

Actin is perhaps best known for its involvement in muscle contraction together with myosin. But actin filaments are also responsible for range of movements in non-muscle cells, e.g. the crawling movement of a cell across a surface. Actin filaments are about 7 nm in diameter and are, like microtubules, an important part of the cytoskeleton [42].
Figure 5.11: Composite image of actin filaments. BSR in green done with 40x 0.95 NA air, confocal in blue done with the same objective and SIM in red done with 63x 1.4 NA oil.

Figure 5.12: Cross section of BSR and SIM corresponding to yellow line in Figure 5.11
5.2. FIXED BIOLOGICAL SAMPLES  

5.2.3 Vimentin

The third component of the cytoskeleton is a so called intermediate filament because its diameter of approximately 10 nm is intermediate between the previously discussed actin filaments and microtubules. Vimentin appears to have little to do with movements of the cells and seems to only provide structure and mechanical strength to the cytoskeleton [43].

Figure 5.14: Composite image of vimentin filaments. BSR in red done with 60x 1.2 NA water and SIM in grey done with 63x 1.4 NA oil.
Figure 5.15: Cross section corresponding to yellow line in Figure 5.14

Figure 5.16: The ROI from the composite image, Figure 5.14. Sub captions indicate the modality of each measurement. BSR and LSM was made with 60x 1.2 NA water and SIM was made with 63x 1.4 NA oil. The FOV is 3×3 µm².
5.2.4 Actin comets *Rickettsia Conorii*

Figure 5.17: Composite image BSR and SIM of Rickettsia comets. BSR in green done with 40x 0.95 NA air objective and SIM in grey done with 63x 1.4 NA oil objective.

Figure 5.18: Cross section of Rickettsia comets corresponding to yellow line in Figure 5.17.
5.3 Measurements of live cells

One of the problems with measuring live cells is photo toxicity which can kill the sample cells. When fluorophores are excited with light they produce reactive oxygen species (ROS) [Wright]. ROS react with proteins, lipids etc. and the fluorophores themselves, which leads to loss of fluorescence signal (photobleaching) and inhibition of the cell cycle or cell death (photo toxicity) [Hoebe]. The production of ROS is highly dependent on the dose of excitation light [Bernas]. The BioAxial method is designed to use as low light dose as possible. Measurements of live cells has been performed with some success. The focus has been to show the ability to measure a specific area of live cells several times with the BioAxial system without adversely effecting or killing them. After BioAxial measurement the condition of the cells are visually examined with a Spinning disc microscope to determine the survival rate. So far no impact has been seen on the cells after BioAxial measurements. Up to four subsequent BioAxial acquisitions of the same group of cells has been made without any sign of harm of the cells. Due to the slow acquisition rate the measurements of live cells has returned quite poor imaging results. For the moment the acquisition time of 20 min for a 2\(^*\)2 mum area is not fast enough to capture the movements of living cells, and the results are blurry.

5.4 Reconstruction

The reconstruction algorithm is based on the Bayesian inverse problem and is a stochastic method that utilise Markov chains. Thanks to the stochastic nature of the Markov chains parallelization of the computation is possible and this greatly reduces the calculation time. The reconstruction is run on Amazon Cloud computing.

The reconstruction is run through the same Matlab GUI called Quickview, discussed earlier. All the micro images from a recent Bead- and Scanner calibration are compressed and uploaded to the Amazon server together with the micro images from the measurement. With the GUI
the user then chooses the number of machines to be used for the calculation and for how long
the calculation should continue. Since the reconstruction is based on a completely stochastic
method each machine receives the complete data set and then runs a number of calculation
steps with a predetermined number of Markov chains. The result of each individual machine
is the averaged with the result from all the other machines to create the final image. A lot of
experimenting with the number of machines needed, as well as the calculation time to reach
convergence, has been done. For the moment no increase in image quality has been found
beyond 8 machines. The calculation time is dependent of the size of the ROI as well as the
step size used for the measurement. A ROI of 2×2 μm² is reconstructed in ≈ 40 min while a
3×3 μm² takes ≈ 1.5 h.

5.5 Artefacts

There are some artefacts sometimes appearing in the reconstructed result that are not fully
understood. One of them seem to have a direct link to primarily the Bead calibration used
while reconstructing and is called vertical line effect. Below an example of a single measurement
of Human Bone Marrow Endothelial Cells (HBMEC) reconstructed with three different Bead
calibrations is presented. One can see distinguished vertical lines in one of the reconstructions
5.20(a), while the other two completely lacks this feature. One of the other two, 5.20(b), seems,
however, more blurry than the last 5.20(c).

![Figure 5.20](image)

(a) (b) (c)

**Figure 5.20**: Same measurement of HBMEC, measured with 40x 0.95 NA air, reconstructed with
three different Bead calibrations. In (a) artefactual vertical lines are clearly visible. In (b) the result
seems to be a bit more blurry than in (c) which is regarded as the best result. FOV 3×3 μm².

Another artefact that is not fully investigated but is thought to be related to the step size of
the acquisition is the so called grid effect and honey comb effect. Both the grid and the honey
comb effect gives the impression that the intensity is restricted to a mesh-like distribute points
in the image, see Figure 5.21. The grid effect has rectangular shaped pixels with intensity.
For the honey comb effect the intensity seems to flow out from the pixels and form hexagonal
shaped holes without intensity.
Figure 5.21: Figure (a) is microtubules, done with 60x 1.2 NA water FOV $2 \times 2 \, \mu m^2$, a grid is visible in the image. Figure (b) is HBMEC, done with 40x 0.95 NA air, FOV $3 \times 3 \, \mu m^2$, a grid is visible but in this case it forms hexagonal shaped holes.
Chapter 6

Conclusions
CHAPTER 6. CONCLUSIONS

A novel technique to obtain super resolution microscopy has been described. With a biaxial crystal, conical diffraction is employed with polarising optics as a mode converter unit to spatially alter the PSF of the illumination light. Four different super-oscillatory intensity profiles are used to obtain sub-diffraction frequency information in four directions of the sample. The versatility of the system has been shown by presenting experimental results of sub-diffraction imaging of a variety of samples with three different objective NA, magnification and immersion medium. The performance is as good and often better than the performance of SIM, which is used as ground truth for correlative measurements, even though a poorer objective in general is used with the BioAxial system. The system can be used with any commercially available confocal microscope with a fibered laser entrance port. The BioAxial module is simply plugged in and with an additional software BioAxial Super Resolution (BSR) is achieved. The main drawbacks of the system in its current developing stage are: a limited FOV, a long acquisition time, a long reconstruction time and occasional artefacts that are yet to be completely understood. All of these limitations are currently investigated and are under development within BioAxial. Improvements are expected soon.

The work at BioAxial has made me realize something that I think would be really interesting and useful for the whole microscope industry. It is the lack of a true and indisputable method of comparing the resolution between different super resolution techniques. For any optical system such a method, of course, exists in the optical transfer function (OTF) and closely related modulation transfer function (MTF), $\text{MTF} = |\text{OTF}|$, which displays the amount of modulation, or ability to reproduce contrast, at specific spatial frequencies. But for super resolution techniques no one seems to have shown any interest in displaying their resolution capabilities with these graphs. I realize that it might be hard to deduce the OTF for such a complex system, but the informative outcome would certainly improve the characterization of the system, not the least for the consumer when choosing which system to buy. Both the SIM and BSR has twice the bandwidth compared to a standard diffraction limited system. This means that they both should have the ability to image structures in the sample at double the cutoff frequency, $f_{\text{cutoff}}$, of the diffraction limit, see Figure 6.1.
Figure 6.1: Descriptive modulation transfer functions for the BSR, SIM and conventional diffraction limited systems. Note: The curves are arbitrary and only intended to give a rough idea about the possible outcome of such a comparison.

However, as we have seen in this report, the BSR system often outshines the SIM resolution-wise, which means that the BSR system must have a better modulation at these particular frequencies. I.e. a higher curve as indicated in Figure 6.1. Perhaps the BSR system has better modulation for all frequencies within the bandwidth, perhaps not. I don’t know, but I think it would be highly interesting, and beneficial for the whole field to investigate.
Appendix A

Polarisation
A.1 Stokes formalism

A general way of describing the polarisation state of a light beam can be done by the Stokes vector. The elements of the vector are called Stokes parameters and are four measurable quantities that describe whether the beam is fully polarised, partially polarised or unpolarised, and how the polarisation is oriented. For quasi-monochromatic light, the definition of the Stokes vector is [44]:

\[
\mathbf{S} = \begin{bmatrix}
S_0 \\
S_1 \\
S_2 \\
S_3 \\
\end{bmatrix} = \begin{bmatrix}
\langle E_x E_x^* + E_y E_y^* \rangle \\
\langle E_x E_x^* - E_y E_y^* \rangle \\
\langle E_x E_y^* + E_y E_x^* \rangle \\
i \langle E_x E_y^* - E_y E_x^* \rangle \\
\end{bmatrix}
\] (A.1)

where \( E_x \) and \( E_y \) are components of the electric field of the beam in the \( x \)- and \( y \)-direction respectively. The brackets \( \langle \rangle \) indicates that the quantities are ensemble averages but, assuming stationarity, ergodicity and monochromatic light they can be dropped.

The first element, \( S_0 \), describes the total intensity of the beam, the second, \( S_1 \), describes the preponderance of linearly horizontally polarised light over vertically, the third \( S_2 \) describes the preponderance of linearly polarised light in the 45\(^\circ\)-direction over \(-45^\circ\)-direction and the forth, \( S_3 \), describes the preponderance of circularly polarised light in the left-handedness over the right-handedness.

The Stokes vector can be rewritten to depict the intensities instead:

\[
\mathbf{S} = \begin{bmatrix}
I \\
Q \\
U \\
V \\
\end{bmatrix} = \begin{bmatrix}
I_0 \\
I_x - I_y \\
I_{45^\circ} - I_{-45^\circ} \\
I_L - I_R \\
\end{bmatrix}
\] (A.2)

The Stokes vector is commonly normalised by dividing each component with the total intensity:

\[
\mathbf{S} = \begin{bmatrix}
1 \\
Q/I \\
U/I \\
V/I \\
\end{bmatrix} = \begin{bmatrix}
1 \\
S_1/S_0 \\
S_2/S_0 \\
S_3/S_0 \\
\end{bmatrix}
\] (A.3)

Below follows a few examples of common polarisations represented with Stokes vectors:

\[
\mathbf{S}_H = \begin{bmatrix}
1 \\
1 \\
0 \\
0 \\
\end{bmatrix} \quad \mathbf{S}_{45^\circ} = \begin{bmatrix}
1 \\
0 \\
1 \\
0 \\
\end{bmatrix} \quad \mathbf{S}_{RC} = \begin{bmatrix}
1 \\
0 \\
0 \\
-1 \\
\end{bmatrix}
\] (A.4)

\[
\mathbf{S}_V = \begin{bmatrix}
1 \\
-1 \\
0 \\
0 \\
\end{bmatrix} \quad \mathbf{S}_{-45^\circ} = \begin{bmatrix}
1 \\
0 \\
0 \\
-1 \\
\end{bmatrix} \quad \mathbf{S}_{LC} = \begin{bmatrix}
1 \\
0 \\
0 \\
1 \\
\end{bmatrix}
\] (A.5)

where the denotation is as follows: \( H \)-Horizontal linear, \( V \)-Vertical linear, \( \pm 45^\circ \)-Linear in given direction, \( RC \)-Right handed- and \( LC \)-Left handed circular polarisation. The degree of polarisation, \( P \), can then be defined as:

\[
P = \frac{\sqrt{Q^2 + U^2 + V^2}}{I} \leq 1
\] (A.6)
Interaction of the light with a medium is mathematically described by a multiplication of the Stokes vector with the so called Mueller matrix of the medium. The Mueller matrix for an optical component describes how the component transforms the different polarisation components of the Stokes vector during interaction. For a linear retarder with retardation $\delta$ oriented with its fast axis at $0^\circ$ the Mueller matrix is [45]:

$$M_{\text{ret}}(\delta, 0^\circ) = \begin{bmatrix} 1 & 0 & 0 & 0 \\ 0 & 1 & 0 & 0 \\ 0 & 0 & \cos \delta & \sin \delta \\ 0 & 0 & -\sin \delta & \cos \delta \end{bmatrix}$$ (A.7)

For a linear retarder oriented with its fast axis in the arbitrary direction $\theta$ one must convert the Mueller matrix with the matrix product:

$$M_{\text{ret}}(\delta, \theta) = M_{\text{rot}}(\theta) \cdot M_{\text{ret}}(\delta, 0^\circ) \cdot M_{\text{rot}}(-\theta)$$ (A.8)

where $M_{\text{rot}}(\theta)$ is the Mueller matrix representation of a rotation around the optical axis of an angle $\theta$:

$$M_{\text{rot}}(\theta) = \begin{bmatrix} 1 & 0 & 0 & 0 \\ 0 & \cos 2\theta & -\sin 2\theta & 0 \\ 0 & \sin 2\theta & \cos 2\theta & 0 \\ 0 & 0 & 0 & 1 \end{bmatrix}$$ (A.9)

### A.2 Jones formalism

There is a more compact and easier way of describing the polarisation state, namely with the Jones vector. The limitation of this formalism is that it only deals with completely polarised light, and hence, unpolarised or partially polarised light cannot be described. The Jones formalism proceeds from the electric vector itself and the Jones vector is:

$$E = \begin{bmatrix} E_x \\ E_y \end{bmatrix} = \begin{bmatrix} E_{0x}e^{i\delta_x} \\ E_{0y}e^{i\delta_y} \end{bmatrix}$$ (A.10)

where $E_x$ and $E_y$ are the complex quantities, $E_{0x}$ and $E_{0y}$ are the field amplitudes in their respective directions and $\delta_x$ and $\delta_y$ is the phases. The Jones vector is commonly normalised by dividing both elements in the vector by the norm of the vector. A few examples of normalised common polarisations is given below:

$$E_H = \begin{bmatrix} 1 \\ 0 \end{bmatrix} \quad E_{45^\circ} = \frac{1}{\sqrt{2}} \begin{bmatrix} 1 \\ 1 \end{bmatrix} \quad E_{RC} = \frac{1}{\sqrt{2}} \begin{bmatrix} 1 \\ -i \end{bmatrix}$$ (A.11)

$$E_V = \begin{bmatrix} 0 \\ 1 \end{bmatrix} \quad E_{-45^\circ} = \frac{1}{\sqrt{2}} \begin{bmatrix} 1 \\ 1 \end{bmatrix} \quad E_{LC} = \frac{1}{\sqrt{2}} \begin{bmatrix} 1 \\ i \end{bmatrix}$$ (A.12)

The equivalent to the Mueller matrix in Stokes formalism is called, simply, the Jones matrix. Here follows an example of the Jones matrix for a linear retarder of retardation $\delta$ [45]:

$$J_{\text{ret}}(\delta) = \begin{bmatrix} 1 & 0 \\ 0 & e^{-i\delta} \end{bmatrix}$$ (A.13)
and similar to the Mueller matrix computation, a retarder oriented with its fast axis at angle $\theta$ has to be transformed with the rotation matrix, $J_{\text{rot}}$, according to the following equation:

$$J_{\text{ret}}(\delta, \theta) = J_{\text{rot}}(\theta) \cdot J_{\text{ret}}(\delta) \cdot J_{\text{rot}}(-\theta)$$  \hspace{1cm} (A.14)

where $J_{\text{rot}}(\theta)$ is given by:

$$J_{\text{rot}} = \begin{bmatrix} \cos \theta & -\sin \theta \\ \sin \theta & \cos \theta \end{bmatrix}$$  \hspace{1cm} (A.15)

A.3 Poincaré sphere

The Poincaré sphere offers an easy way of visualising the polarising effect of a retarder on incident light. Every possible pure polarisation can be represented by a point in the surface of the Poincaré sphere. The north and south pole represent right and left circularly polarised light, respectively, and the equator represents different orientations of linearly polarised light. The points in between represent various states of elliptically polarised light. A line along the one of the longitudes has constant orientation but varying ellipticity and vice versa for a line along one of the latitudes. Points below the surface of the sphere represent partially polarised states.

The influence of a linear retarder on a beam of given polarisation is depicted by a rotation $\delta$ in the left hand direction (clockwise) on the Poincaré sphere. The axis of rotation is defined by the orientation of the fast axis of the retarder, $\theta$, see Figure A.1.

![Figure A.1: The Poincaré sphere. Original image can be found in [45].](image)

If for example horizontally polarised light is incident on a quarter wave plate ($\delta = \pi/2$) oriented with its fast axis at $45^\circ$, the effect of the retarder can easily be seen by consulting Figure A.1. By rotating $\pi/2$ in the clockwise direction from the starting point $H$ around the $45^\circ$-axis we end up in position $L$, i.e. circularly polarised in the left hand direction, which is a well known result with a quarter wave plate. Should the retarder for instance be oriented at $-45^\circ$ the result is given by a $\pi/2$ rotation in this axis and we end up in right-hand circularly polarised light, and so on.
Bibliography


