



The University of
Nottingham

**Improved resolution and signal-to-noise
ratio performance of a
Confocal Fluorescence Microscope**

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ABSTRACT

A culmination of theory, techniques and devices stemming from a wide variety of sources and disciplines, optical microscopy presents vast possibilities for visualisation of small structures. One of the most fundamental yet significant optical microscopy techniques is Confocal Fluorescence Microscopy (CFM). CFM is studied here by analysing its performance with respect to the two most important metrics - Signal-to-noise ratio and 3D optical resolution. Several authors have commented on the inherent inefficiency of imaging systems such as CFM to utilise the available light when providing resolution beyond the well-known diffraction limit, primarily due to the precise mechanisms that help realise the resolution gain in the first place. In CFM, the detection pinhole is the key mechanism that helps realise up to 1.4 times resolution improvement over conventional wide-field microscopy techniques by trading off SNR.

First, an investigation of the inherent SNR-resolution trade-off in a CFM system is studied; the impact of the detection pinhole geometry on the performance of a CFM is examined by means of an effective trade-off curve. Using alternative pinhole geometries in conjunction with new detection schemes, it is next shown how performance gains are realised in both the lateral and axial directions.

Examined next is a recently developed detection scheme called subtractive imaging; wherein a special annular pinhole is used to divide the confocal point spread function signal into two detectors. By using fast point detectors in place of CCD arrays, it is shown how using numerical optimisation yields an optimum “differential pinhole” to achieve considerable 3D resolution gains over conventional (circular pinhole based) CFM systems. By examining the trade-off curves it is also shown that the proposed design is able to offer simultaneous and maximum performance gains up to a considerably high SNR in comparison to conventional (circular pinhole) based CFM systems.

Lastly, the work will propose the use of a deconvolution technique and an alternative detection scheme to demonstrate substantially higher improvements in the quality of images acquired by a CFM system. Image reconstruction is a tried and tested image post processing strategy to realise super resolution. An image reconstruction technique, based on an expectation maximisation maximum likelihood (EM-ML) algorithm is used in conjunction with array detectors to demonstrate enhanced resolution and noise performance of a CFM system. The point scan method used here renders the algorithm slow with long run times. To mitigate this, structured illumination is used to show how similar resolution gains in the array detector based CFM systems could be realised but in a much shorter time.

LIST OF PUBLICATIONS

Journal Papers

R. Kakade, J. G. Walker and A. J. Phillips, “Optimising performance of a confocal fluorescence microscope using a differential pinhole”, IOP Measurement science and technology, vol. 27 pp 1-8, 2016

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LIST OF ACRONYMS

AO	Adaptive Optics
APD	Avalanche Photodiode
CARS	Coherent Anti-Stokes Raman Scattering
CCD	Charge Coupled Device
CFM	Confocal Fluorescence Microscopy
EM	Electron Microscopy
EM-ML	Expectation Maximisation Maximum Likelihood
ER	Equivalent Radius
FLIM	Fluorescence Lifetime Imaging
FLIP	Fluorescence Loss in Photobleaching
FRAP	Fluorescence Recovery after Photobleaching
FRET	Fluorescence Resonance Energy Transfer
FT	Fourier Transform
FWHM	Full Width Half Maximum
GN	Generalised Normal
HELM	Harmonic Excitation Light Microscopy
I ² M	Image Interference Microscopy
MPM	Multiphoton Microscopy
NSOM	Near-field scanning optical microscopy
ODP	Optimised Differential Pinhole
OTF	Optical Transfer Function
PALM	Photo-activated Localization Microscopy
PMT	Photomultiplier Tube
PSF	Point Spread Function
SIM	Structured Illumination Microscopy
SMLM	Single Molecule Localisation Microscopy
SNR	Signal-to-noise ratio
SPIM	Selective Plane Illumination Microscopy
SPRM	Surface Plasmon Resonance Microscopy
SSIM	Saturated Structured Illumination Microscopy
STED	Stimulated Emission Depletion
STORM	Stochastic Optical Reconstruction Microscopy
SWFM	Standing-Wave Fluorescence Microscopy
TIRF	Total Internal Reflection Fluorescence Microscopy
WFM	Wide-field Fluorescence Microscopy

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CHAPTER 1

INTRODUCTION

1.1 Motivation

This project deals with improving the resolution as well as the noise performance of one of the most important optical microscopes for bio-imaging - Confocal Fluorescence Microscope (CFM); more specifically, in optimising the trade-off between the two. In general, optical or light microscopy refers to any imaging system employing visible light and a lens system to image minute details of a specimen. Critical in the study of life sciences, optical microscopy provides a non-invasive way to view biological samples with the naked eye [1]. The resolution of a microscope can be defined simply as the smallest distance at which two points on the object can be seen separately. For a conventional widefield microscope this is diffraction limited to $\sim 200\text{nm}$ which greatly limits its applicability in bio-imaging. Several advanced high resolution techniques such as stochastic optical reconstruction microscopy (STORM) [2], structured illumination microscopy (SIM) [3], photo activated localisation microscopy (PALM) [2] and stimulated emission depletion microscopy (STED) [4] have been developed over the past decade that surpass the classical diffraction limit.

However, they are expensive, complex and often demand long specimen exposure times making them often ill-suited for live cell imaging. A simpler, more robust and less complex alternative is CFM which is well known for its superior optical sectioning (ability to produce clear images of focal planes deep within a thick sample) capabilities. CFM involves

illuminating the object with a focussed beam and projecting the light collected at the conjugate plane onto a point detector. This enables improving the resolution by a factor of $\sqrt{2}$ over widefield microscopes.

The SNR-resolution trade-off is of great importance to bio-imaging applications where the aim is to image the sample using as little light as possible without considerably sacrificing image quality. This work details novel detection schemes and image reconstruction algorithms to realise 3D resolution improvement and improved noise performance in CFM systems. The performance improvements will be demonstrated through computer simulations.

1.2 Thesis layout

This chapter outlines the principal motivation behind the research undertaken and summarises the original contributions to knowledge made (section 1.3). The thesis is organised as below with each chapter containing a short summary of the key points discussed therein-

To set the context to the work undertaken, a historical timeline of the development of optical microscopy is first presented in chapter 2. Next, detailed discussions on the key concepts in microscopy – principally resolution and signal to noise ratio are made. The importance of fluorescence microscopy to bio-imaging with its underlying principles is then explained. Finally, a review and comparison of the many advanced microscopy techniques in existence today is presented.

Chapter 3 describes the imaging process of a CFM, discussing the key principles and analytical expressions required for numerical modelling of the

same. The impact of the pinhole geometry on the 3D resolution and SNR performance will be examined by means of an effective trade-off curve.

Next, in chapter 4 an investigation into employing an alternative detection scheme in conjunction with new pinhole geometries will be presented to show how performance gains in either direction can be realised. Computer simulation results will be used to demonstrate the improvement in image quality.

In chapter 5 a new confocal imaging technique called subtractive imaging is introduced. By analysing the pinhole geometry an optimum pinhole profile is presented that achieves substantial resolution gains in both lateral and axial directions. The impact of background noise on the system performance will also be discussed. Furthermore, using a recently developed pupil filter – fan shaped pupils - it will be shown how the axial resolution of an optimised differential pinhole based CFM can be further enhanced.

Chapter 6 demonstrates how, using array detectors, 3D resolution gains can be achieved using a stochastic deconvolution approach – maximum likelihood (ML) processing. A comparison of ML processing with other deconvolution methods is first made to demonstrate its suitability for CFM image reconstruction. Next, through computer simulations it is shown how ML processing of data acquired using array detectors substantially improves image quality over both conventional (pinhole based) CFM systems and those that employ ML processing. The chapter will also show how using structured light, the ML processing run time can be reduced substantially with almost no loss in image quality. Finally chapter 7 will draw conclusions and identify future work.

1.3 Original Contributions to Knowledge

This section highlights the original contributions to knowledge made in this thesis.

- The CFM system performance has been characterised by means of an effective SNR-resolution trade-off. Using pixel array based detectors in conjunction with alternative non-circular pinhole geometries, it has been demonstrated using computer simulations how lateral/axial resolution as well as SNR gains over conventional CFM systems can be realised.
- A new detection scheme that employs a “differential pinhole”, based on the recently introduced concept of subtractive imaging for CFM systems, is proposed to show how considerable improvement in image quality over conventional a CFM system can be realised. Initial results were published (conference paper) in the Springer Proceedings in Physics [5].
- By parameterising the “differential pinhole” an optimum pinhole geometry is obtained that offers simultaneous and maximum resolution gains in both lateral as well as axial directions over conventional CFM systems. Improvements are demonstrated through computer simulations. Findings have been published in IOP Measurement Science and Technology [6] .
- An array detector based CFM system has been developed that employs maximum likelihood based image reconstruction algorithm. Comparative analysis between the new method and existing pinhole detector based CFM systems is used to demonstrate the technique’s superiority. A paper has been submitted for review to IOP Measurement Science and Technology [7].

- Using an array detector and structured illumination, it has been demonstrated how similar resolution and SNR improvements in a CFM can be realised using ML processing but achieving the same at much higher speeds.

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CHAPTER 2

LITERATURE REVIEW

2.1 Development of Optical Microscopy

Historically, the Dutch scientist Anton van Leeuwenhoek (1632-1723) is considered to be the inventor of the light microscope. However, it was Robert Hooke (1635-1703), an English scientist, who is largely credited with being the first to publish research based on using an optical microscope [1]. In his book titled *Micrographia* he was able to accurately describe the fruiting structures of living moulds using an optical microscope [2]. Hooke achieved this by using a more complete imaging system with two lenses as opposed to Leeuwenhoek's single double convex lens microscope. In the 1800s several scientists then attempted to alleviate problems of lens aberrations; in 1830, Joseph Jackson Lister, for example, demonstrated how using multiple lenses could reduce spherical aberrations [3]. Another problem - chromatic aberration was partially resolved by Italian scientist Giovanni Amici by introducing a mirror based achromatic reflecting microscope [4]. It was however only much later, in 1886, that the chromatic aberrations were substantially reduced. This was achieved through a collaborative effort between Ernst Abbe, Carl Zeiss and Otto Schott who introduced apochromatic lenses [5].

Work then began on improving the resolution of the optical microscope and in 1890, after a decade of work on developing the diffraction theory for image formation, Abbe proved that collecting a large cone of light (i.e. larger numerical aperture, NA) from the object resulted in higher resolution. Subsequently he introduced several oil immersion microscope

objectives (NA = 1.4) into the Carl Zeiss microscopes as well as matching high NA condenser to demonstrate 200 nm resolution with visible light [3]. In this period, Abbe published several articles comprehensively detailing his image formation theory for the optical microscope. The Abbe diffraction theory also unravelled the dependence of resolution on other factors such as wavelength and coherence of light. This allowed obtaining images of previously unknown bacteria. Another important contribution was made by August Kohler who introduced a new optical setup using what was subsequently named Kohler illumination [6]. Here the specimen received uniform illumination resulting in high image contrast and reduced image artefacts.

By the turn of the 20th century the fundamental theory and operating principles behind optical microscopes were established. The technological improvements spanning 400 years has enhanced our understanding of how this fascinating instrument works as well as make important advances in medicine, chemistry and biology [2, 3]. 1903 saw the invention of an ultramicroscope by Richard Zsigmondy (Nobel Prize in chemistry recipient 1962) which allowed observing tiny suspended particles (colloids) as small as 4nm although they are several orders below the wavelength of visible light. It was basically a form of dark field microscopy using an intense source of light projected normal to the optical axis of the microscope.

Another technical challenge in microscopy was to be able to image inherently low contrast specimens such as unstained thin tissues. These thin specimens being phase objects, they cannot be observed using conventional microscopes; human eyes are insensitive to phase variations. A brilliant

scientist – Fritz Zernike (Nobel Prize in Physics recipient 1953) overcame this problem, inventing the first phase-contrast microscope which enabled mapping of phase variations of the light interacting with the specimen into detectable intensity changes [7]. It is important to note that major advances in life sciences and medicine have not only been brought about by improvement in optical microscopy but also from parallel development of techniques such as sample fixation, staining, embedding and sectioning [8].

The early 2000s saw the growth of adaptive optics (AO) technology which has considerably minimised effects of lens aberrations and facilitated improving performance of conventional as well as various super resolution techniques in existence today. A recent article by Martin [9] contrasts and compares various AO methods. Furthermore, development of high-efficiency detectors such as charged couple devices (CCDs) have allowed imaging entire samples all at once yielding high SNR images essential to acquiring high resolution data.

2.2 Resolution

In its simplest form, resolution is the smallest discernible distance between two points on a specimen. Higher resolution translates to being able to see the specimen in higher detail. A small point object when imaged with a light microscope will, due to diffraction, produce a ‘blot’ which is referred to as a point spread function (PSF). The diffraction phenomenon, as observed by Abbe, depends on the numerical aperture of the objective lens and the wavelength of light used.

2.2.1 Point Spread Function

A very simple way to visualise a point spread function is to imagine the pattern created when a single drop of ink is gently dropped on a piece of white paper. The PSF is thus an impulse response of the optical system. PSFs are typically circularly symmetrical in the lateral (x-y) direction (fig 2.1(a) [10]) and broadened in meridional plane of the optical axis (z) (fig 2.1(b) [10]).

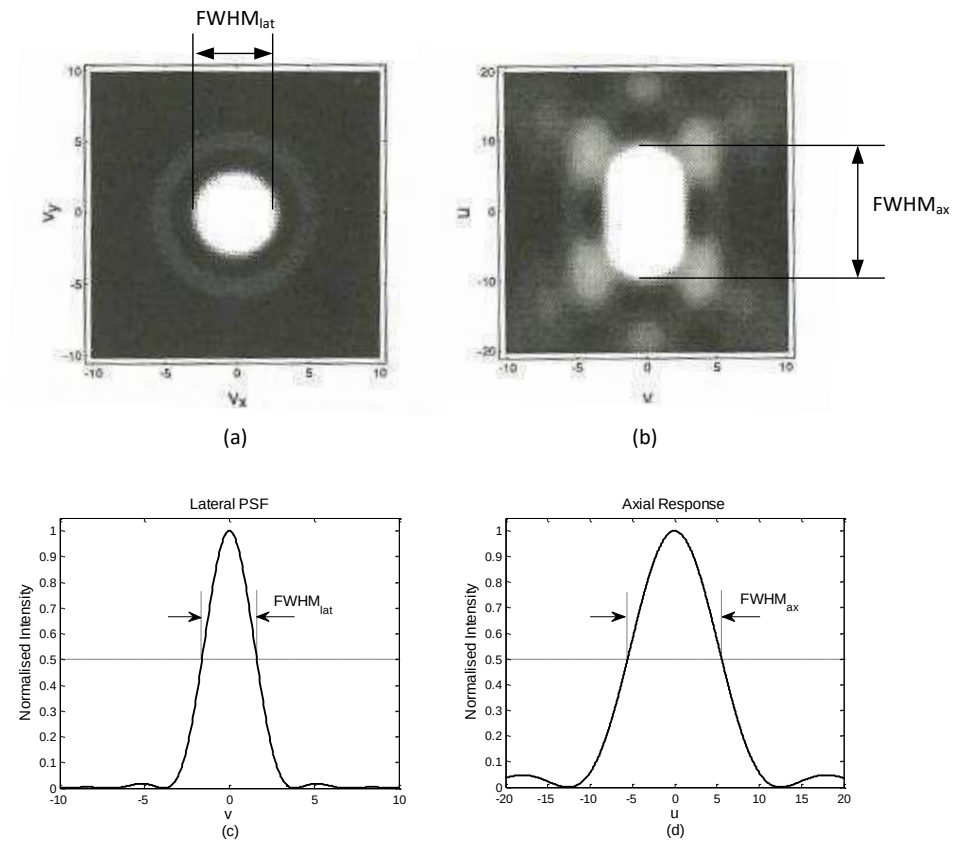


Figure 2.1: Point spread function (PSF) of a wide-field microscope as seen in the (a) x-y (focal plane) plane [10] and (b) x-z plane (meridional plane of the optical axis) [10]. (c) PSF seen as 1D cross section of (a) along x/y directions. (d) Axial (z-direction) response. FWHM_{lat} and FWHM_{ax} refer to the lateral and axial full-width-half-maximum widths respectively. The intensities have been normalised by the intensity at the centre of the PSF.

The circularly symmetric intensity distribution of the in-focus PSF is often referred to as an Airy pattern. Most optical systems, being linear shift-invariant, can be idealised to have a position-independent PSF[11]. If a specimen is visualised as an array of several point emitters each with its own

strength, then its image can be considered equivalent to the sum of corresponding PSFs. This property greatly simplifies optical theory, in that the imaging operation can be described as the convolution of the object with the system's PSF.

PSF of a conventional microscope

For a simple widefield microscope, using circular lens pupils, the intensity distribution of the infocus PSF I_{lat} and axial response I_{ax} can be computed using equation 2.1 and 2.2 respectively [12],

$$I_{lat}(v) = I_o \left| \frac{J_1(v)}{v} \right|^2, \quad (2.1)$$

$$I_{ax}(u) = I_o \left| \frac{\sin\left(\frac{u}{4}\right)}{\frac{u}{4}} \right|^2. \quad (2.2)$$

Here v is the radial displacement in optical units and u is the axial displacement in optical units related to the real radial displacement r and real axial displacement z by equation 2.3 and 2.4 respectively. I_o is the intensity at the centre of the diffraction pattern and J_1 is the first order Bessel function of the first kind. Although, equation 2.1 and 2.2 can be readily deduced (see [10]) from Kirchhoff's diffraction formula under the Fresnel approximation, it is not derived here for brevity. The optical units are defined by,

$$v = krNA, \quad (2.3)$$

$$u = kz \eta \sin^2\left(\frac{\alpha}{2}\right), \quad (2.4)$$

Where k is the wavenumber related to the wavelength of light λ as given by equation 2.5. NA is the numerical aperture of the lens given by equation 2.6.

$$k = \frac{2\pi}{\lambda}, \quad (2.5)$$

$$NA = \eta \sin(\alpha), \quad (2.6)$$

here η is the refractive index of the medium separating the lens and the sample and α is the lens half-angle.

2.2.2 Resolution criteria

Although, Ernst Abbe and Lord Rayleigh were the first to clearly formulate a general resolution criterion, several criteria have since been introduced to describe the fundamental goal of resolution viz., to discriminate the constituent features of the object. The Nyquist theorem, as a resolution criterion, for example, can prove to be very useful in describing the ability of a microscope technique to discriminate structures of an unknown specimen. At other times, a measure of the smallest detectable feature size may be of greater importance or to set a value to the smallest detectable observation volume. A criterion based on measuring the 3D observation volume is particularly useful to life sciences e.g., to measure how well a microscope system can discriminate 3D structures in a single cell. The ultimate choice of a resolution criterion is problem based. Having a universal resolution metric that is directly transferable between techniques is a complicated and possibly unachievable task as recently concluded by Justin et al [13]. Some of the most commonly used criteria are described next.

1. Full-width-half-maximum: In a microscope system, the image of a two-point object will appear as two closely spaced Airy disks. It is evident that the sharper an Airy disk is (i.e. smaller diameter), the more separated it will appear from the other Airy disk. This concept has been generalised in the resolution criterion based on full width half maximum (FWHM) of the PSF

[14] as shown in fig 2.1 (c) and (d). FWHM is the diameter of the PSF at which its intensity is one-half of the maximum intensity. Generally, two object points are said to be resolved if the distance between their PSFs is greater than the FWHM. It should be noted that, depending on the optical system, FWHM has different values in the focal plane (infocus x-y plane) and in the meridional plane (plane along the optical axis z).

2. Rayleigh Criterion: As resolution is often defined as the minimum separation between two points, the term is commonly discussed in context of two-point objects. The Rayleigh criterion defines resolution as the separation between two equal intensity point objects at which the maximum intensity of the PSF from one object coincides with first minimum of the PSF from a second object. For a widefield system the lateral resolution given by,

$$r_{lat} = \frac{0.61\lambda}{NA} \quad (2.7)$$

which in optical units is 3.83. This is demonstrated in figure 2.2. At this separation, the system exhibits a 26.4% contrast (viz., defined as the difference between the central maximum and minimum intensity expressed as a percentage of the maximum intensity [15])

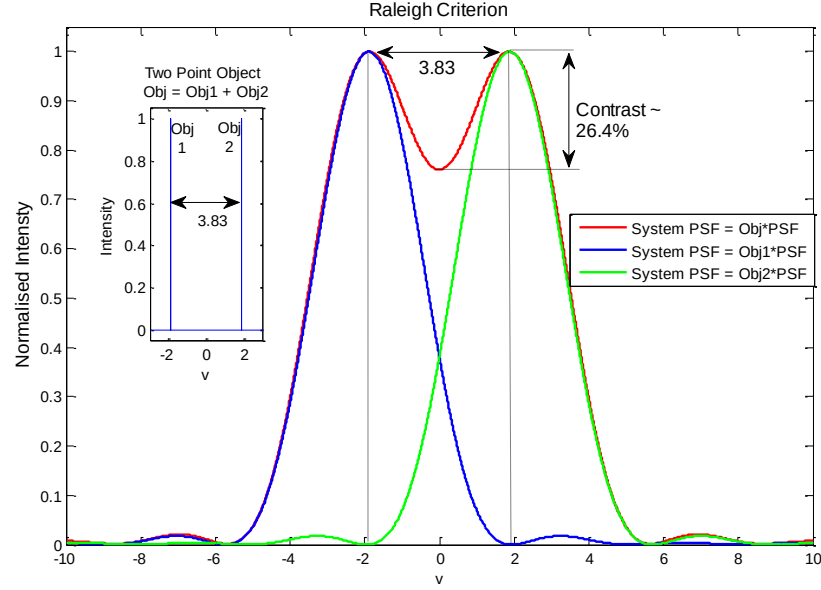


Figure 2.2: Widefield microscope response (PSF) to a two-point object. Here (*) represents a convolution operation.

Axial resolution can be similarly defined as the object separation at which the maximum of the axial response from one object coincides with the minimum of the axial response of the other object [16, 17]. This is given by,

$$r_{ax} = \frac{2\lambda}{NA^2} \quad (2.8)$$

3. Sparrow criterion: The Sparrow limit is simply the distance between two identical point objects at which a dip half way between them disappears in the superimposition of the two images [18, 19]. The Sparrow limit is approximately 0.78 times the Rayleigh limit and is given by equation 2.9 [2]. Figure 2.3 [20] shows the Rayleigh and Sparrow limits for the infocus PSF response to two identical point objects.

$$r_{lat}^{sparrow} = \frac{0.47\lambda}{NA} \text{ and } r_{ax}^{sparrow} = \frac{1.56\lambda}{NA^2} \quad (2.9)$$

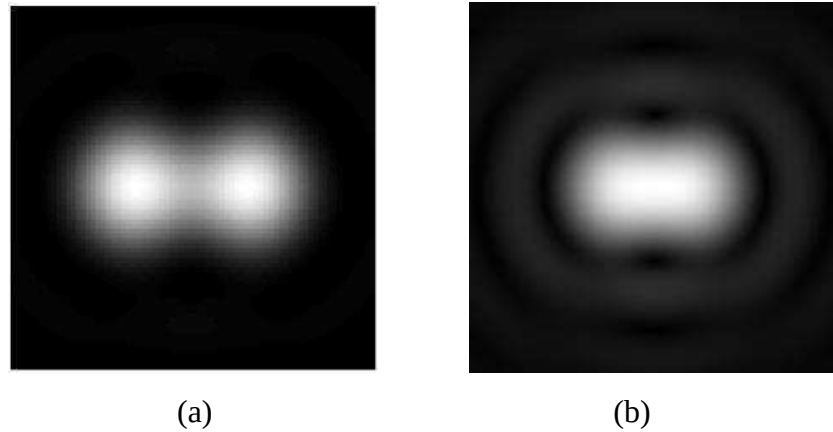


Figure 2.3: Infocus PSF responses of a conventional microscope imaging two identical point objects [20] at (a) Rayleigh criterion and (b) Sparrow criterion

Both the Rayleigh and Sparrow criteria fail to account for undesired PSF sidelobes (visible in figure 2.2). These sidelobes can often appear accentuated by optical engineering approaches adopted in microscope systems [21]. These resolution criteria are thus not absolute and can only be considered as ‘soft’ resolution limits. A ‘hard’ resolution approach that addresses this is the Abbe limit.

4. Abbe Limit: In order to understand Abbe’s resolution criterion, picture an arbitrary specimen made up of periodic structures (frequency m) as shown in figure 2.4. Any such specimen contains information and imaging fundamentally relates to the process of the information flow through a lens system. Since for a coherent imaging system, the light distribution in the back focal plane is but the Fourier transform (FT) of the specimen illumination Fourier transformation can be used to define the information content of the specimen. Redrawn from Gu’s work [22] figure 2.4 shows how the FT of the specimen function yields a transfer function for a single lens system. As seen from the figure for a 1D specimen light gets diffracted into 3 components one

along the optical axis and the other two at an angle θ to it (note for simplicity of explanation only the first harmonics are considered). This angle is related to the spatial frequency m of the specimen as $\theta = m\lambda$ which tells us that finer gratings would lead to a smaller diffraction angle. Now only light incident within the lens' acceptance cone (i.e. $\leq \alpha$) would contribute to the final image.

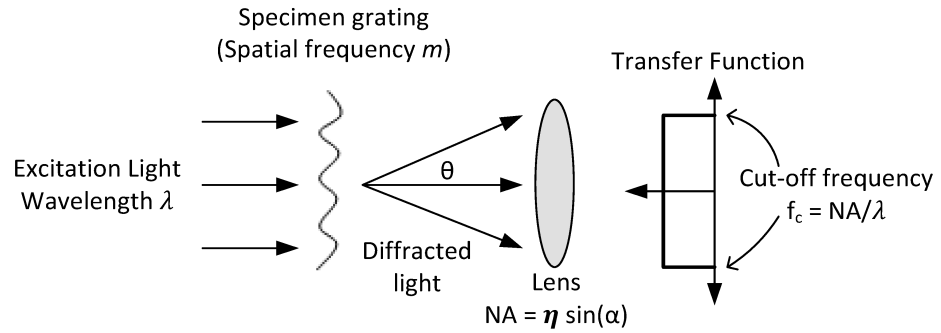


Figure 2.4: 1D Specimen consisting of a sinusoidal grating of spatial frequency m being illuminated by uniform illumination of wavelength λ . Abbe limit states that such a specimen is 'resolved' if the angle of the diffracted orders of light $\theta \leq \alpha$. Note that any specimen, periodic or not, can be described by a sum of sinusoidal waves.

Thus the maximum specimen spatial frequency f_c that can be captured occurs when $\theta = \alpha$. Thus $f_c = \alpha/\lambda$. Any specimen (or specimen features) with spatial frequencies higher than this cut-off would not be imaged rendering the lens to a low pass filter of sorts. This cut-off frequency represents the Abbe limit. The efficiency of an optical system lies in its ability to transmit different spatial frequencies and is described by its transfer function [22]. In the case of an incoherent imaging system, such as in confocal fluorescence microscopy, wherein the point spread function represents an intensity variation, the transfer function is referred to as Optical Transfer Function (OTF). In the lateral and axial directions the Abbe limit for a widefield fluorescence microscope is given by equation 2.10 and 2.11 respectively [21],

$$r_{lat}^{Abbe} = \frac{0.5\lambda}{NA} \quad (2.10)$$

$$r_{ax}^{Abbe} = \frac{2\lambda}{NA^2} \quad (2.11)$$

2.2.3 Terms related to resolution

In imaging, terms such as ‘magnification’, ‘sensitivity’, ‘precision’ and ‘sampling’ are often confused with ‘resolution’. A brief discussion on this will be made next to clarify some common misconceptions.

1. Sensitivity vs resolution: Sensitivity refers to the ability to simply detect say a single fluorescent molecule under a microscope, but does not convey the dimension of the molecule. Thus single molecule sensitivity could be achieved by a microscope system producing at best a fuzzy image (poor resolution).

2. Sampling vs resolution: Magnifying the image of a molecule can be optically achieved to any degree, but this does not improve our ability to see greater detail. At best it preserves it. Detectors (human eyes, point detectors or even CCD cameras) take discrete measurements from the image into intensity values with each detector element holding one measurement [23]. These measurement (or ‘sampling’) points correspond to (but not limited to) nominal coordinates of the object being imaged. The Nyquist sampling theorem dictates that at least two detector elements are needed to sample the finest detail in the object, in order to capture all information in the object [24]. Meeting this criterion is achieved through magnification, but the sampling process does not increase the information capacity (or resolution) of the optical system [15].

3. Precision vs resolution: If the points in an object are sufficiently far apart then the individual centres of each point can be localised with high precision. Thus a microscope system can have precision far greater than its optical resolution [25, 26]. For example localisation error on single molecules can be <10nm on a 200nm resolution system. Some microscopy techniques that offer high localisation precision will be discussed in section 2.4.

4. Depth of Field vs Depth of Focus: Depth of field is the distance from the closest object plane which is simultaneously in focus with another in-focus object plane farthest away [27]. Depth-of-focus differs from the depth of field in that it is defined over the image space. Although the image plane can be assumed to represent a very thin specimen section it is true that every point in its space is diffracted above as well as below this plane. Young [28] formulates depth-of-focus Δz to be given by,

$$\Delta z = \frac{\lambda}{4\eta \left(1 - \sqrt{1 - \left(\frac{NA}{\eta} \right)^2} \right)}. \quad (2.12)$$

This indicates that, neglecting lens aberrations, at high NA the in-focus and the out-of-focus points can be tolerated closer to one other and hence an extended depth of focus. This limited depth of focus does not pose a big problem to thin specimen imaging but is a real challenge for thicker specimens.

2.3 Signal-to-noise ratio (SNR)

In optical microscopy the signal to noise ratio is defined as the ratio of the detected signal to the total noise i.e. from background as well as from the uncertainty in counting intrinsic quantum mechanical events (e.g. photon

counting at the detector). Of the various noise sources that exist (electronic noise, dark current noise, auto-fluorescence out of focus light etc.) photon shot-noise imposes a fundamental limit on performance in most fast optical systems. Governed by the Poissonian distribution function, the shot noise follows the property of having its mean equal to the variance [29], $p(n, \lambda)$, written as,

$$p(n, \lambda) = \frac{\lambda^n e^{-\lambda}}{n!}, \quad (2.13)$$

denotes the probability of n occurrences when the average expectation number of events is λ [30]. For a signal composed of an average of N photons the best achievable SNR is given by equation 2.14, which indicates that in a shot noise limited system the best achievable SNR is equal to the square root of the total detectable photons. The actual SNR is often lower due to background signals originating from the environment and or the detector itself. SNR is also influenced by the detector gain characteristics and inefficiencies linked to signal processing of the acquired photons,

$$\text{SNR}_{\text{best}} = \frac{\text{Mean}}{\sqrt{\text{Variance}}} = \frac{N}{\sqrt{N}} = \sqrt{N}. \quad (2.14)$$

The impact of shot noise on the PSF is shown in figure 2.5. By considering the noisy image, the inability of Rayleigh/Sparrow resolution criterion to yield a ‘hard’ resolution limit can be seen clearly.

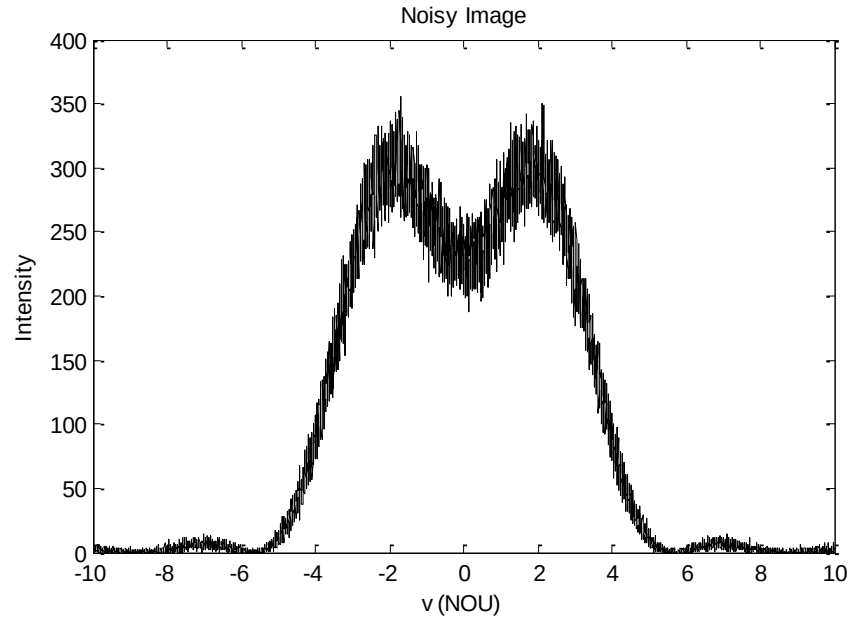


Figure 2.5: Image of the two point object (shown in fig 2.2) with shot noise. Owing to the ambiguity in contrast seen here, Rayleigh and Sparrow criterions fail to offer a reliable measure of resolution.

Using a finite size detection pinhole, as in confocal microscopy can eliminate most of the background noise and improve the SNR (more on this in chapter 3). Using a cooled CCD, thermal effects such as dark currents can also be minimised. Readout noise depends on the camera readout rate. Most scientific cameras operate between 20kHz to 500kHz at which the readout noise is small enough to be ignored [31]. For video-rate cameras this can be a significant problem. It might appear from equation 2.14, that by increasing the incident laser power (i.e. photons in illumination) a higher SNR can be achieved. This isn't completely true as in fluorescence microscopy a phenomenon called fluorophore saturation causes the fluorophores' emission efficiency to drop after receiving a certain amount of laser power. Furthermore photobleaching can also take place wherein the fluorophore permanently loses its ability to emit photons. Such effects set practical limits on the achievable SNR.

2.4 Fluorescence Microscopy

On the resolution spectrum, at one end Positron Emission Tomography (PET), Magnetic Resonance Imaging (MRI) and Optical Coherence Tomography (OCT) allow imaging details no smaller than $\sim 1\text{mm}$, $\sim 100\mu\text{m}$ and $\sim 10\mu\text{m}$ respectively. At the opposite end, Electron Microscopy offers nanometer resolutions but suffers from limitations such as sample fixation and radiation damage (explained further in section 2.5). Set in the middle of this resolution spectrum is Fluorescence Microscopy (FM) offering a range of spatial resolutions. FM has proven to be a powerful and versatile imaging tool for biologists with its ability to tag virtually every molecular protein. The fluorophore molecules being very bright are easily distinguishable from other background signals, thus facilitating high-contrast imaging. Other forms of microscopy generally lack this level of specificity but provide other useful information; molecular orientation for example can be studied through polarisation microscopy. Unstained cells can also be studied using dark-field, differential interference contrast and phase contrast microscopy. While the latter two are inherently unsuited for imaging thick samples; dark field exposes the sample to excessive light, thus risking cell damage. The next section will discuss the principle of fluorescence before shifting focus on understanding the imaging principle of a CFM.

2.4.1 Principle of Fluorescence

Absorption and re-emission of light by specimens is a consequence of a well-known physical phenomenon termed as fluorescence [32]. First observed by Stokes in 1852 it was not until 1930 that fluorochromes were experimentally used to mark biological samples such as bacteria, protozoa and other

pathogens [29, 32]. Certain samples like chlorophyll auto-fluoresce naturally while others need to be treated with synthetic fluorescing chemicals emitting light when excited through radiation of matching wavelength [33]. The principle of fluorescence is explained in figure 2.6 below. As demonstrated a fluorophore atom's electron upon excitation (a) gets promoted to an elevated energy state (b). For this migration excitation energy corresponding to the difference between the two energy levels needs to be pumped. From quantum physics we know that this corresponds to a specific excitation wavelength λ_{ex} . On relaxation (c) not all of the excitation energy is released in fluorescence rather some of the energy is lost through “vibrational relaxation” meaning less energy to fluoresce and consequently a longer emission wavelength λ_{em} . This observed wavelength shift is called Stokes shift (see figure 2.6 (e)). The emitted fluorescence from a biological specimen can be detected both as an image and photometric data. This allows carrying out quantitative as well as qualitative analysis on the inner specimen cell structure. With the ability to single out desired molecules within a specimen, fluorescence has enabled high contrast, highly versatile and highly specific imaging [34]. This has been the motivation behind the onset of a new kind of microscopy - fluorescence microscopy - a virtually indispensable imaging technique to any biologist today.

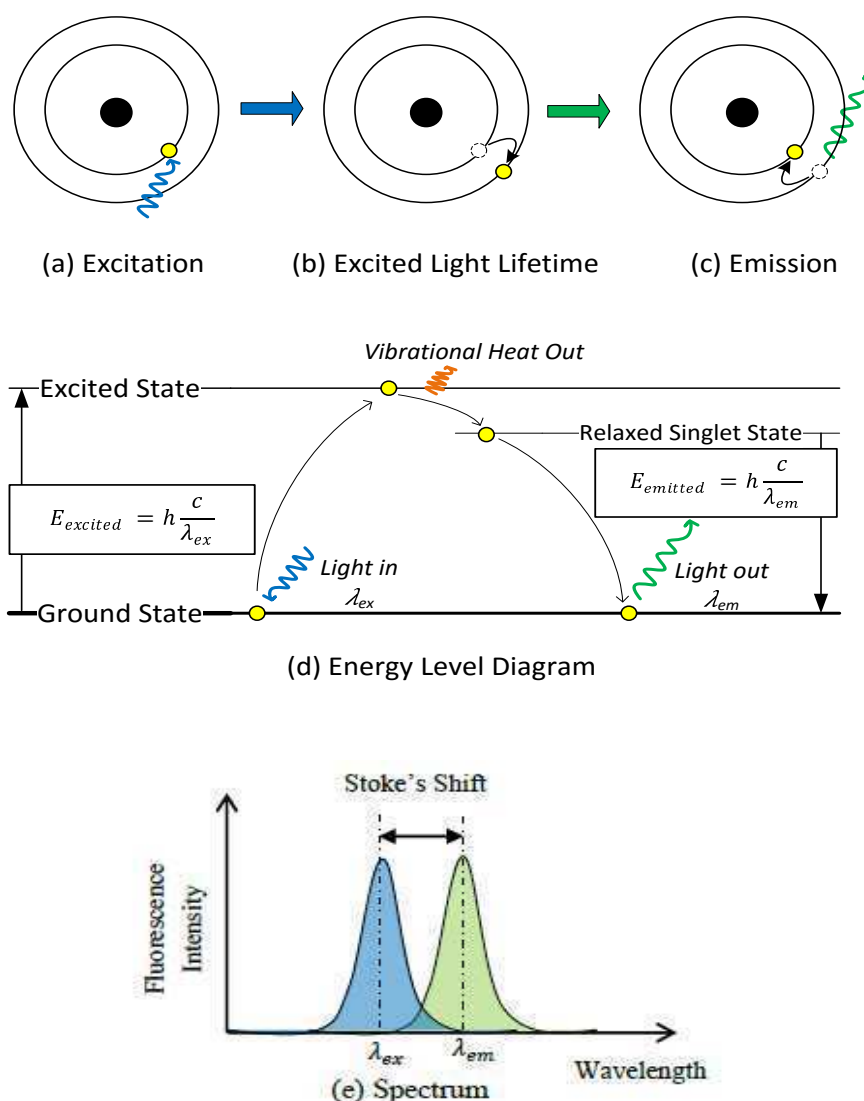


Figure 2.6: Three stage fluorescence process shown (a) Absorption of excitation energy by fluorophore atom, (b) electron jumps to higher energy state and (c) rapid fall back to ground state through photon emission. (d) is an energy level representation of (a), (b) and (c) while (e) shows the excitation and emission spectra.

2.4.2 Significance of photobleaching

Photobleaching, also known as optical bleaching or fading is the destruction of a fluorophore through a photochemical process brought about by excessive exposure to light [35]. Figure 2.7 [35], showing an example of this phenomenon, demonstrates the significant loss of fluorescence (as intensity) experienced by a fluorophore over time (lapses shown (a) to (f)). It can also be noted here that some fluorophores are more resilient than others to bleaching.

Note in the above example that the blue and red (Alexa Fluor 350 and 568) fluorophore bleach out much more quickly than the green (Sytox Green) fluorophore.

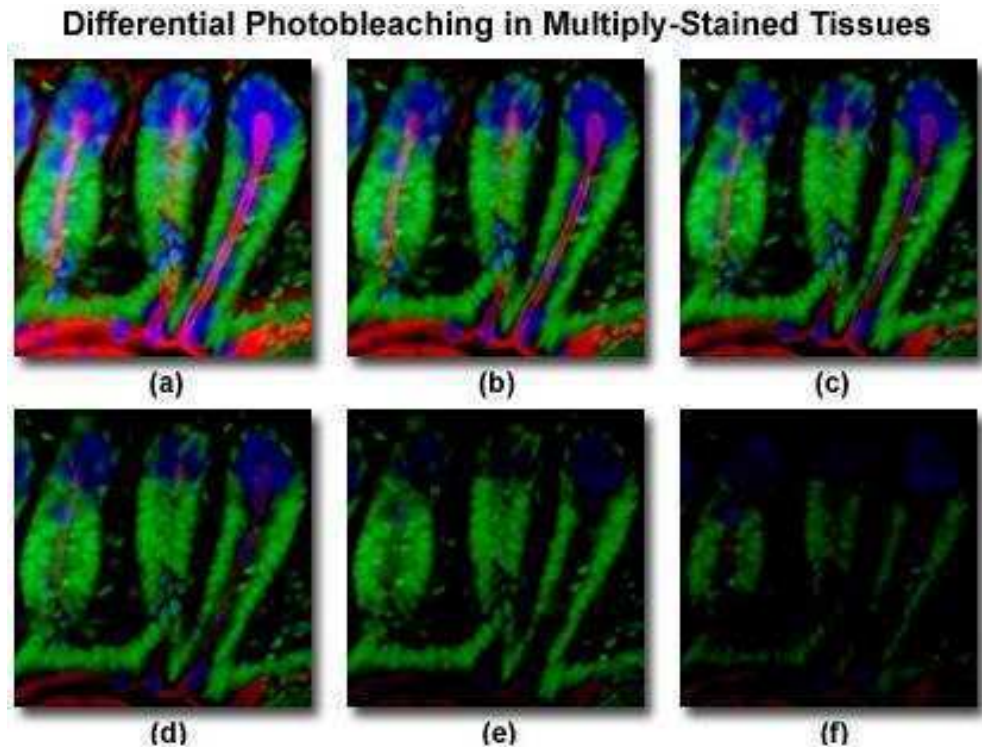


Figure 2.7: Photobleaching observed in a series of digital images (a)-(f) of a thin (16 μ m) slice of mouse intestine. The specimen is stained with three different fluorophores to highlight different structures. Each image is recorded after a 2 minute interval. The Sytox green stained nuclei shows more resilience to photobleaching [35]

In fact, photobleaching can be exploited to gather very specific information e.g., ascertaining the lateral diffusion rate [35-37] of a chosen fluorophore and motion tracking of fluorescently labelled molecules [36]. As CFMs allow control of the region of illumination, they are ideal for photobleaching techniques such as Fluorescence Recovery after Photobleaching (FRAP) and Fluorescence Loss in Photobleaching (FLIP), both of which are widely used today for diffusion rate measurements. Other approaches are Fluorescence

lifetime imaging (FLIM) and Fluorescence resonance energy transfer (FRET) [34].

FRET occurs when the energy in a fluorophore gets transferred to its neighbouring fluorophore. This phenomenon occurs when the two fluorophores are oriented in a dipole-dipole coupling orientation and overlap spectrally [38]. The energy transfer efficiency being inversely dependent (to the power of 6) [38] on the fluorophore separation, minute protein-protein interactions can be measured in live cells. Unfortunately though, hypothetical fluorophore pairs do not always exhibit FRET in live cells. Until substantial improvements in imaging methods and formulation of new FRET pairs are made, the FRET technique continues to see limited application in FM.

FLIM is based on measuring the excitation lifetime of a fluorophore [39] as opposed to the conventional counting of the photons emitted by the excited state. FLIM is capable of detecting several spectrally overlapping fluorophores [40] making the approach well suited to measuring FRET [41]. However, the approach requires heavy mathematical processing, is technically demanding and offers limited resolution.

2.5 High Resolution Microscopy Techniques

Following the development of the first electromagnetic lens in 1926 by Hans Busch [42], two German scientists - Ernst Ruska (Nobel Prize recipient 1986) and Max Knoll invented the first electron microscope [7]. Electron microscopy (EM), a technique that uses high energy electron beams, although offering a much higher resolution than optical microscopy enabling biologists to examine otherwise invisible cell structures, exposes samples to high

vacuum and invasive treatment prior to observation [27]. Also, EM demands specimen dehydration and thin sectioning during sample preparation which can be technically challenging and prone to artefacts [43], rendering EM unfavourable for the study of live biological processes. Therefore, the development of high resolution and high contrast optical microscopes has taken centre stage in bio-imaging. Witnessing an enormous growth in the past decade or so, optical microscopy has found applications for micron and submicron level examinations [27]. For example the Ultra Resolution Imaging (URI) system developed recently features an enhanced spatial resolution of less than 150 nm with improved contrast [27, 44].

Among the many developments that have ensued is fluorescence microscopy which allows individual structures, specific proteins and genetic loci to be labelled making it a principal choice for most biological applications. Traditionally the diffraction limit viz. approximately half the wavelength of light limits the spatial resolution to under 200 nm but today through fluorescence microscopy nearly a tenfold improvement in spatial resolution has been achieved. As a direct consequence of extensive and on-going research linear techniques namely structured illumination, 4Pi and I⁵ microscopy have been developed. Some nonlinear techniques such as Stimulated Emission Depletion (STED), Stochastic Optical Reconstruction Microscopy (STORM) and Photo-activated Localization Microscopy (PALM) offering 10-20 nm resolutions [27, 44, 45] have also been developed. Yuval et al. [44] present a clear picture of the classification of optical microscopy technologies in existence today; an adaption of which is presented in figure 2.8.

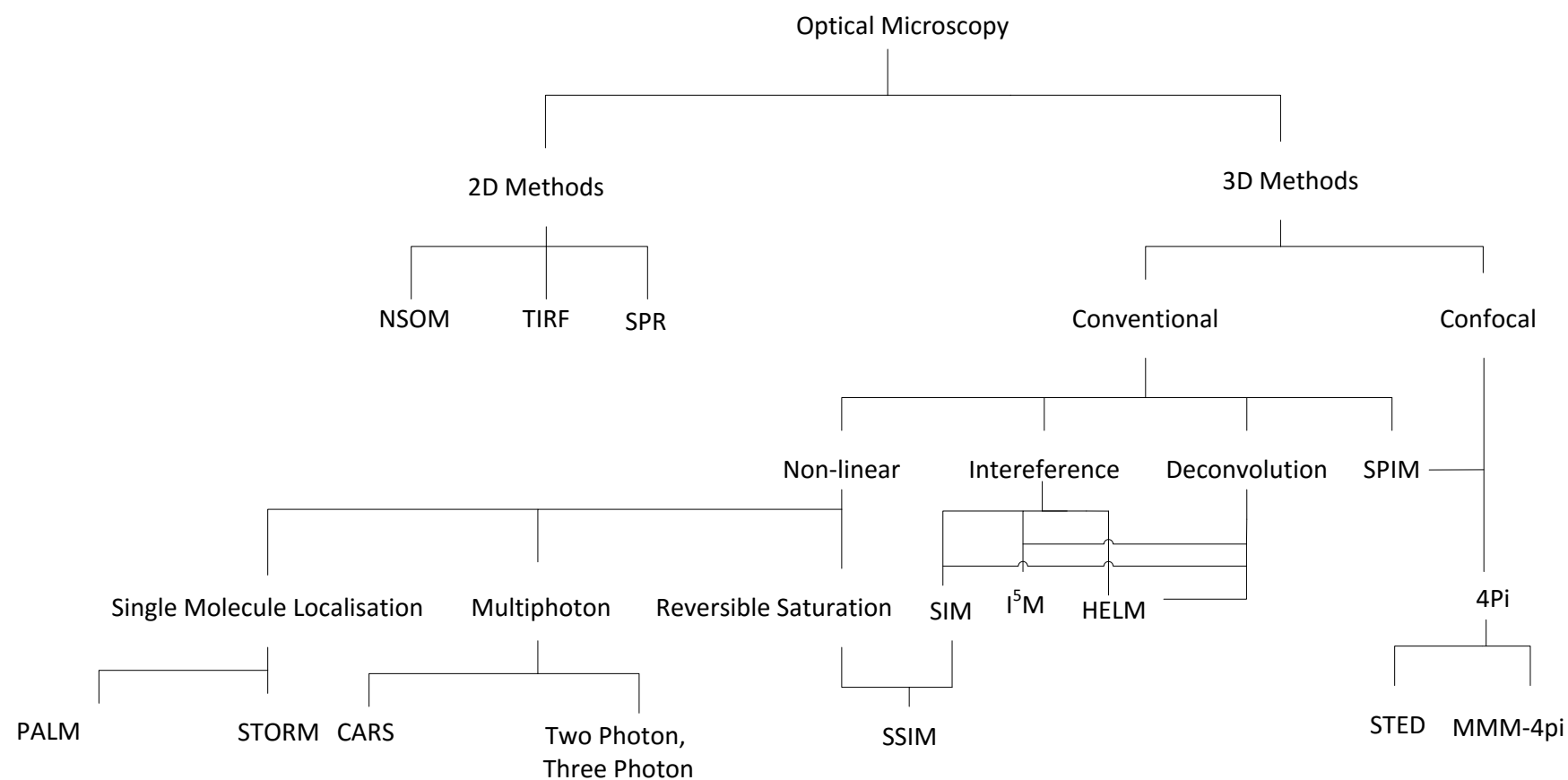


Figure 2.8: Map of available super resolution optical microscopy methods(adapted from [44])

2.5.1 Conventional (Wide-field) and Confocal Microscopy

The humble wide-field (WF) microscope shown in figure 2.9(a) involves illuminating the specimen (through an objective lens) with light from a xenon or mercury source and viewing the image directly on a photographic film or another capture device. Its inability to reject out of focus light severely limits its applications in fluorescence microscopy. Confocal Fluorescence Microscopy (CFM) developed soon after provided $\sqrt{2}$ resolution improvement by using a single point laser illumination that was scanned across the specimen and subsequent detection thorough a pinhole (see fig 2.9(b)). CFM operation and imaging principles will be discussed in further detail in chapter 3.

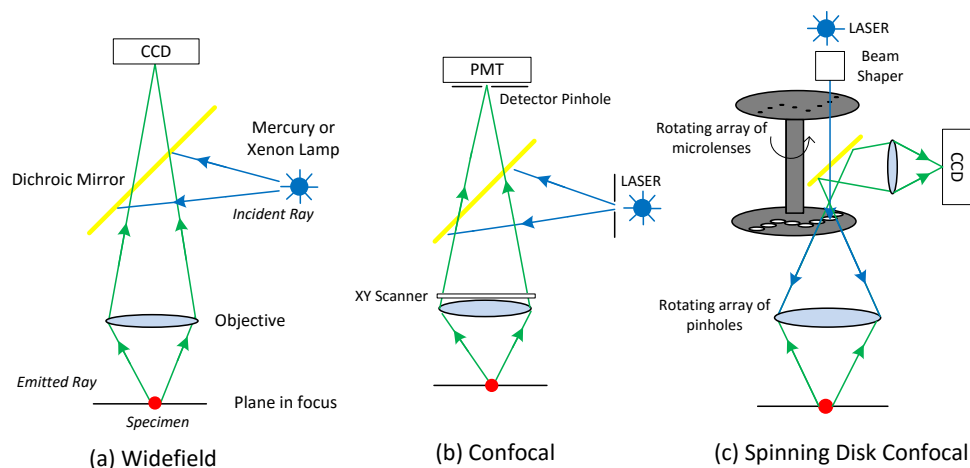


Figure 2.9: Schematics (adapted from [46]) of a (a) Widefield, (b) scanning confocal and (c) spinning disk confocal microscope systems. Illumination beams are shown in blue and emitted beams in green.

The most important feature of a CFM is its excellent out-of-focus light rejection making it an instrument of choice for microbiologists [16, 47]. One drawback of CFM is its relatively slow data acquisition speed. Using a punched spinning-disk pinhole aperture (see fig 2.9(c)) speed improvements have been made but at the expense of slightly reduced resolution [46], and the need for high precision in pinhole placement and design[48]. Selective Plane Illumination Microscopy (SPIM) – a new method further increased imaging

efficiency relative to CFM with resolution of $\sim 1\ \mu\text{m}$ for large specimens [49]. This was achieved by employing a semi-confocal illumination allowing the entire specimen to be imaged at once. The onset of digital image processing has led to the development of numerous deconvolution methods to remove image blur. They are especially useful when dealing with small specimens imaged in low light conditions. Deconvolution methods can be classified into deblurring and restoration algorithms. While the former treat the various optical sections as separate 2D images (and is thus susceptible to mishandling blurred light) the latter iteratively estimates an object that, when convolved with the point-spread function of the microscope, could produce the image data [50]. A detailed discussion of deconvolution methods is given in Chapter 6.

2.5.2 Interference based Microscopy

It is possible to generate a patterned illumination at the specimen plane by the interference of two sources. The resulting fluorescence emission will hold higher resolution information than captured using conventional microscopy. By employing two objectives, one in front of and the other behind the specimen, the effective numerical aperture can be increased, improving the resolution even further. A similar effect is observed when fluorescence collected from two high numerical aperture objectives is interfered at the CCD plane. This is the principle for Image Interference Microscopy (I^2M). Owing to the fact that interference pattern intensity attenuates heavily through thick samples this technique is restricted to thin specimens. 4Pi microscopy, one other interference based method, uses a laser source in a confocal mode to provide axial resolution in the 100 nm range although like I^2M provides no significant lateral resolution improvement. Another method also based on structured-light

is Standing-Wave Fluorescence Microscopy (SWFM) [51]. SWFM uses all the excitation light as an axially varying standing wave to achieve similar axial resolution gains. Gains in the lateral direction can be realised using techniques such as structured illumination microscopy (SIM) [52] and harmonic excitation light microscopy (HELM) [53]. In SIM, the specimen is illuminated with sinusoidal strip patterns of high frequency. A schematic of a SIM system is shown in figure 2.10 (a). The fluorescent specimen containing fine structures interferes with the high frequency patterns to produce coarse Moire fringes (fig 2.10a(b) [54]) at the image plane.

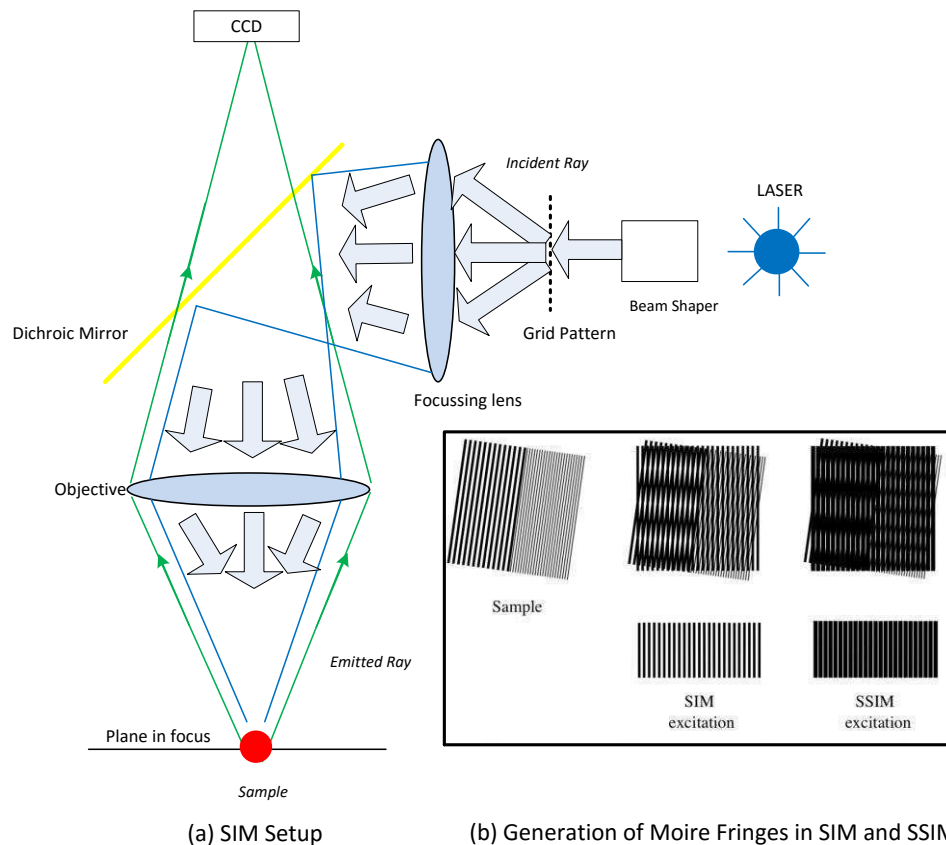


Figure 2.10: (a) Schematic of a Structured Illumination Microscope (SIM) showing how a grid pattern diffracts the excitation beam and then focuses back the diffracted beams onto the sample to create a sinusoidal pattern. The interference of this high frequency sinusoidal pattern with the sample generates the Moire fringes shown in (b) [54]. These are then imaged onto a CCD camera. Saturated-SIM pattern is also shown here for comparison.

These Moire fringes transfer the otherwise non-observable high-frequency information of the sample into a low-frequency region which is observable through the microscope. By changing the orientation (typically 9-15 times [54]) of the illumination pattern, several 2D images of the specimen are recorded which are then processed via various minimisation and cross-correlation algorithms to generate an image with 100 nm lateral resolution [55]. SIM is suitable for most structures in live-cell imaging owing to the relatively small number of orientations needed to obtain a high resolution 2D image [56]. In 3D-SIM, three-beam interferences and an axially oriented excitation pattern, is used to obtain axial resolutions up to 250 nm (twice as good as widefield) [21]. Harmonic Excitation Light Microscopy (HELM), which deploys four beams instead of two to interfere at the CCD plane, yields ~100 nm resolution in both axial and lateral directions [53].

2.5.3 Non-Linear based Microscopy

Unlike the previous methods wherein resolution is enhanced through structured illumination coupled with image processing, non-linear methods have a different approach. Two-photon microscopy, which is a form of Multiphoton Microscopy (MPM), is one such example. The two-photon excitation phenomenon, the theory for which was established by Goppert-Mayer in 1931 [57], arises from the simultaneous absorption of two photons in a single quantised event. A necessary condition is that the two absorbed photons must have about twice the wavelength as that required for one-photon excitation. As two-photon excitation relies on simultaneous absorption, the resulting fluorescence emission varies with the square of the excitation intensity. This quadratic relationship between the excitation and emission intensities offers

many advantages to two photon microscopy. It allows one to reach extremely small fluorescing focal volumes (i.e. very little absorption in out of focus planes) resulting in enhanced spatial resolution and lower susceptibility to photodamage and photobleaching for the fluorophores outside the focal volume [2]. Also the absence of a detection pinhole offers increased design flexibility. Another advantage of MPM techniques is the wider separation between the excitation and emitted light which makes it easier to suppress the excitation light. MPM is vastly used in imaging thick samples as it minimises absorption of shorter wavelength excitation energy in the immediate surroundings. Limitation of MPM is that it is only suitable for fluorescent imaging (reflected light imaging not possible) and that it is not suited for imaging highly pigmented cells which absorb near-IR light [2].

Another non-linear method, Coherent Anti-Stokes Raman Scattering microscopy (CARS), allows real time label-free imaging of thick samples [58]. Being a label-free technique, CARS permits imaging of samples that cannot be stained due to unavailability of appropriate dye. In the CARS process, a pump beam (frequency ω_p) and a Stokes beam (frequency ω_s) interact with the sample via a wave mixing process. When the difference of the two frequencies (Raman shift, $\Delta\omega = \omega_p - \omega_s$) matches the sample molecular vibration frequency a strong anti-Stokes signal ($\omega_{as} = 2\omega_p - \omega_s$) is generated which allows molecular detection with high specificity. Although it offers good optical sectioning capabilities (penetration depth up to 0.4mm) is less suitable for detecting low concentration molecules.

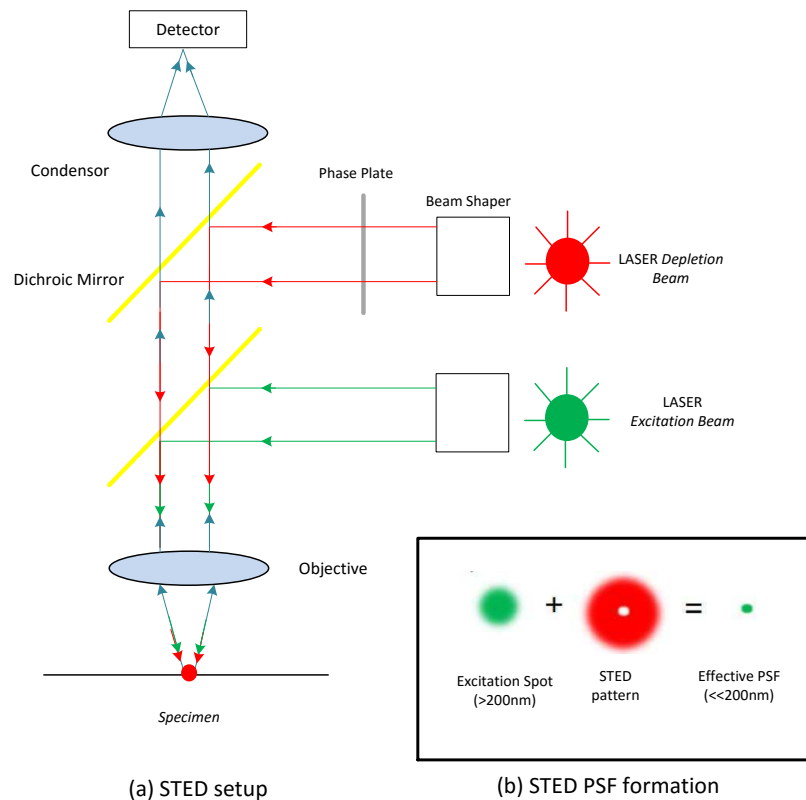


Figure 2.11: Principle of Stimulated Emission Depletion (STED) microscopy. (a) The system requires two lasers – one for the excitation and the other for de-excitation. A STED pattern (doughnut shaped) is formed using a phase plate. (b) A sub-diffraction spot at the specimen plane by super-imposition of the emission and STED pattern (adapted from [59]).

Yet another non-linear method – Stimulated Emission Depletion Microscopy (STED) uses two pulsed lasers in a manner to ensure the total fluorescing specimen volume is kept very small (see figure 2.11 (b) [59]) providing images with resolution of ~ 30 nm [59]; a large improvement over interference based methods. A schematic of the setup and operating principle is shown in figure 2.11 (a). This technique developed by Stefan Hell (Nobel Prize in Chemistry 2014) in 1994 [60], has enabled the study of translucent specimens previously visible only through EM. STED is relatively fast (video rate speeds) compared to all other super-resolution techniques when imaging small specimen areas. However when scanning large pixel areas, STED requires longer scan periods

which adds to the risk of specimen photobleaching [61]. Using a process called Reversible Saturable Optical Fluorescence Transitions (RESOLFT) the effect of high power STED beam can be mitigated. RESOLFT uses the STED pattern to switch fluorophores into the off state rather than into stimulated emission. RESOLFT depletion beam uses up to ~100 times less laser power than STED depletion beam [62].

Yet another non-linear technique that is gathering momentum is an extension of the SIM technique discussed in section 2.5.2 called Saturated Structured Illumination Microscopy (SSIM) [63]. By using structured light illumination from two powerful interfering beams, almost all fluorescent molecules in the illumination beams saturate, leaving only small volumes unsaturated at the shadows of the interference pattern. Using more and more powerful interfering beams, these unsaturated volumes can be made much smaller than any PSF width. 50 nm resolution has been demonstrated using SSIM [64]. The high intensity beams required to obtain such high resolution renders it unsuitable for bio-imaging. Using photoswitchable fluorophores however, the non-linearity required for SSIM can be achieved at low intensities [65] but at the cost of requiring up to 5 times more images to achieve the same resolution.

2.5.4 Single Molecule Localisation Microscopy (SMLM)

SMLM differs from all other fluorescence imaging systems in that, as the name suggests, the image is built up literally one molecule at a time. SMLM works on the principle that although the diffraction limit prevents two objects closer than 250 nm to be distinguished, their centres can be located with nanometer precision [66, 67] limited only by the system SNR. This is achieved by

stochastically turning on fluorescence from the specimen one fluorophore at a time to form a single camera recording and repeating the same till all activated fluorophores are bleached and all the several thousand molecules have been imaged. Then, all the images are summed up to yield a sub-diffraction sized final image. SMLM relies on the temporal separation of fluorophore emission achieved either through successive binding of individual fluorophores to the specimen or by simply alternating their dark and fluorescent states. For this purpose SMLM requires photoconvertible dyes (or fluorescent proteins) whose emission wavelength can be optically converted from one wavelength to another or photoactivatable proteins whose fluorescence can be switched on or off. This has led to development of two techniques in 2006 – PALM [68, 69] and STORM [70] respectively.

Depending on the number of photons collected, SMLM techniques are capable of localising molecules with ~20 nm resolution [59, 71]. The principle of PALM and STORM is shown in figure 2.12 (adapted from [59]). More recently, the principle has been extended to conventional organic dyes, which under proper buffer conditions have been shown to photoswitch robustly [72]. As SMLM allows super-resolution imaging of the molecular distribution it can be used to provide information about molecular interactions [72, 73]. Molecular density measurements can also be used to obtain 3D information from 2D projections [74]. One drawback of molecular counting arises from the fact the fluorophores often ‘blink’ resulting in multiple observations of the same molecule. Thus sufficient care must be taken during molecule identification.

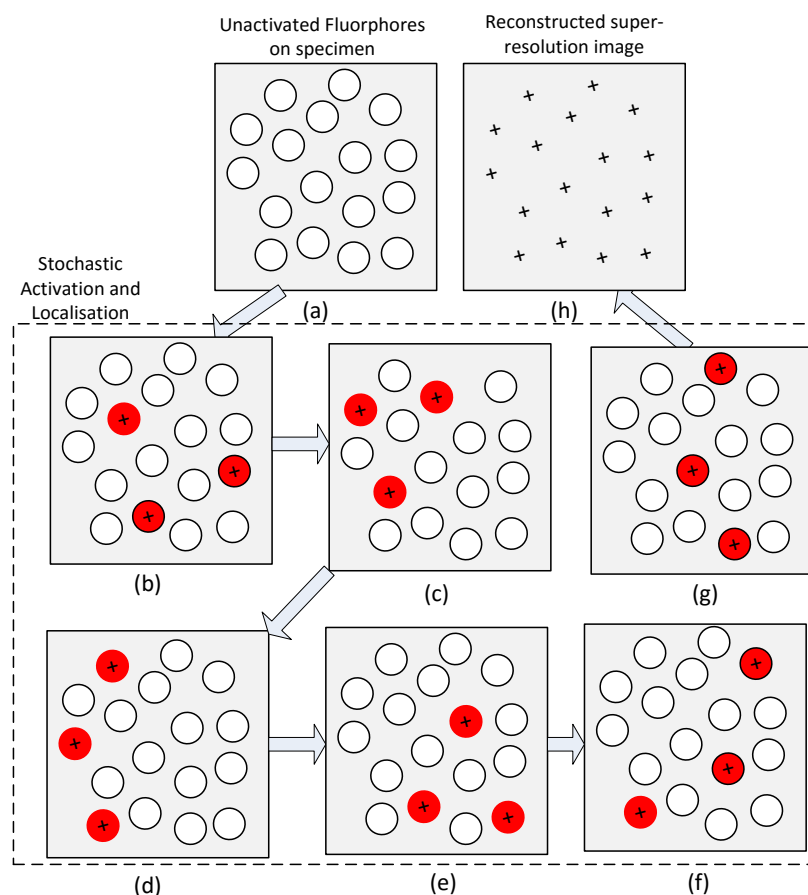


Figure 2.12: PALM/STORM concept of high-precision localisation. (a) The fluorophores are initially in the non-fluorescent state. They are then stochastically photoactivated individually or in sets as shown ((b)-(g)). Using PSF fitting, the centroids of each PSF is determined (marked as +). Sequentially all the fluorophores are activated to obtain localisation of each fluorophore in the structure. (h) Finally all the positions are superimposed to reconstruct the final sub-diffraction limit image.

2.5.5 High resolution 2D Microscopy

Near-field scanning optical microscopy (NSOM) and Total Internal Reflection Fluorescence Microscopy (TIRFM) are two methods that yield very high resolution in 2D measurements. NSOM has been shown to provide optical resolution of up to 30 nm [58] in the lateral direction. It achieves this by measuring near-field evanescent waves using a probe close to the sample surface, discounting thereby the need for focusing using lenses. A TIRFM on the other hand achieves 100 nm axial resolution by using an evanescent wave

to selectively illuminate and excite fluorophores in a confined region of the specimen immediately adjacent to the glass-specimen medium interface [75]. The evanescent wave is generated only when the incident light is totally internally reflected at the glass-specimen medium interface. Image generation in NSOM and TIRFM require moving the scanning probe very close to the surface of the sample which can be technically challenging. Another method based on creating an evanescent wave is Surface Plasmon Resonance microscopy (SPRM) [76] which differs from TIRFM setup in that the glass is coated with a metal layer. SPRM is capable of measuring very small refractive index (10^{-9}) [77] variations by determining the intensity changes as a function of impinging angle.

To summarise section 2.5, there exist an array of super-resolution techniques that offer sub-diffraction limit imaging each with its own set of advantages and disadvantages. Table 2.1 (end of the section) summarises the resolution limits of all these. It must be noted that differences in the various super-resolution techniques ultimately governs how well a technique is suited for answering a specific biological question. It is up to the user to utilise their knowledge of the specimen to choose a technique. As of today no ideal system exists that is capable of offering 3D nanometer scale resolution with multicolour capabilities and high-speed data acquisition for live cell imaging[43].

Table 2.1: Comparing resolution of different Microscopy methods [43-45, 64, 78].

Microscopy Technique	Resolution Limit (μm)	
	Lateral (XY)	Axial (Z)
Wide field (WF) Microscopy	0.25	1.0
Confocal Laser Scanning Microscopy (CLSM)	0.18	0.50
4Pi	0.20	0.09
I ⁵ M	0.23	0.09
HELM	0.10	0.10
Single Molecule Localisation - PALM, STORM	0.02	-
Structured Illumination – SIM	0.10	-
Saturated Structured Illumination Microscopy (SSIM)	0.05	-
2 Photon Microscopy	0.20	0.40
Stimulated Emission Depletion (STED)	0.03	0.05
Total Internal Reflection Microscopy (TIRFM)	0.23	0.10
Near Field Scanning Optical Microscopy (NSOM)	0.03	0.01

2.6 Choosing the right resolution criterion

Generally resolution is defined from the theoretical approach of the Rayleigh criterion but is almost always defined by the Full Width Half Maximum (FWHM) measure in practical approaches. Defining a single resolution metric to evaluate performance of every microscopy technique is a non-trivial and complicated task. SIM and widefield techniques for example can be compared using FWHM as their images are of similar type and dynamic range. But the same measure applied to STED images has a tendency to underestimate the

resolution owing to the inherent low dynamic range of the STED images. Even the Fourier analysis based Abbe criterion tends to underestimate STED's demonstrated ability to resolve highly-contrasted structures. This is again due to its limited dynamic range and low signal at high frequencies. Getting agreements across criteria for SMLM proves even more challenging due to, amongst other reasons, the extremely high accuracy of localising individual fluorophores that makes label density a limiting factor.

Given the above issues, inspecting the PSF response (and performing a Gaussian fit in practice) to compute the FWHM is currently the best approach to compare different microscopy techniques. For this reason, in this thesis FWHM as a resolution metric shall be predominantly used, although the thesis shall introduce an alternate metric to analyse performance of CFM systems with novel detection schemes.

2.7 Chapter Summary

Optical microscopy has advanced enormously in the last 400 years. Super-resolution techniques have given biologists an unprecedented ability to image down to the nanoscale. Today not only can one investigate complex biological structures but also observe them change with time. And yet, the theory and principles behind optical microscopy have essentially remained unchanged. Some of the key points presented in this chapter are summarised as follows -

- The performance of an optical microscopy system is governed not only by its ability to see specimen structures in high detail but also its ability to deliver a high signal to noise ratio.

- Fluorescence microscopy has proven to be an indispensable tool in bio-imaging due to its high specificity and minimally invasive approach.
- Amongst the various resolution metrics developed to date, the PSF width measured at half of its maximum (FWHM), is the most widely used in practical approaches.
- Amongst the various noise sources, photon shot noise sets the fundamental limit to achievable SNR in a fluorescence microscope.
- Several super-resolution optical microscopes have evolved which surpass the diffraction limit, making nanometer scale resolutions a reality. Most notable of those are non-linear techniques such as STED and NSOM as well SMLM techniques such as PALM and STORM.
- CFM, one of the fundamental bio imaging system, offers several advantages, many of which make it more suitable for live cell imaging than the super-resolution techniques; CFM has the ability to provide unusually clear examination of thick light-scattering specimens[79] with high contrast, offers simple implementation and is capable of scanning over large areas of the specimen [16]. It also allows electronic magnification and most importantly is capable of scanning in the axial direction.

The next chapter will detail the imaging process of a CFM system and discuss in further detail its advantages and limitations.

2.8 References

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CHAPTER 3

CONFOCAL FLUORESCENCE MICROSCOPY

Confocal Fluorescence Microscopy (CFM) allows one to image detailed cellular structures as well as create magnificent 3D functional images to examine on-going reactions within the structure [1] and profile 3D surfaces in a non-invasive manner [2]. Following its inception in the 1960s [1] other important technologies such as the laser and reliable computers provided improvements in CFMs making them highly sophisticated imaging instruments with superb imaging capabilities. Compared with conventional widefield microscopy, where the entire sample is illuminated, in CFM only a single point (or an array of such points) is excited at a time. By subsequently scanning all the available points a complete image is created. The advantage here lies in restricting light from out of focus points into the lens objective which improves image sharpness and contrast [2]. Today's confocal systems facilitate data acquisition software for complex techniques such as FRET, FRAP and spectral deconvolution. The following sections will explain the imaging principle of a modern CFM.

3.1 Modern CFM imaging

The underlying principles of CFM were first drawn out by Minsky in 1960 while working as a post-doctorate at Harvard University [3]. Fundamentally his method was based on projecting the final image of the specimen onto a conjugate focal plane (see figure 3.1) where a pinhole restricts light from regions outside the objective lens' principal focus.

In an epi-fluorescence laser scanning confocal microscope laser light is emitted through a beam collimator which is located in a plane conjugate to the desired specimen plane and a pinhole aperture aligned ahead of the detector (generally a photomultiplier tube). The intensity of the laser light may be regulated using acousto-optical tunable filters (AOTFs) or a combination of AOTFs and neutral density filters. An AOTF is an electro-optical device that operates as an electronically tunable excitation filter to simultaneously modulate the intensity as well as wavelength of multiple laser lines from one or more sources. AOTFs rely on specialised birefringent crystals whose optical properties are a function of the acoustic pressure applied on their surface.

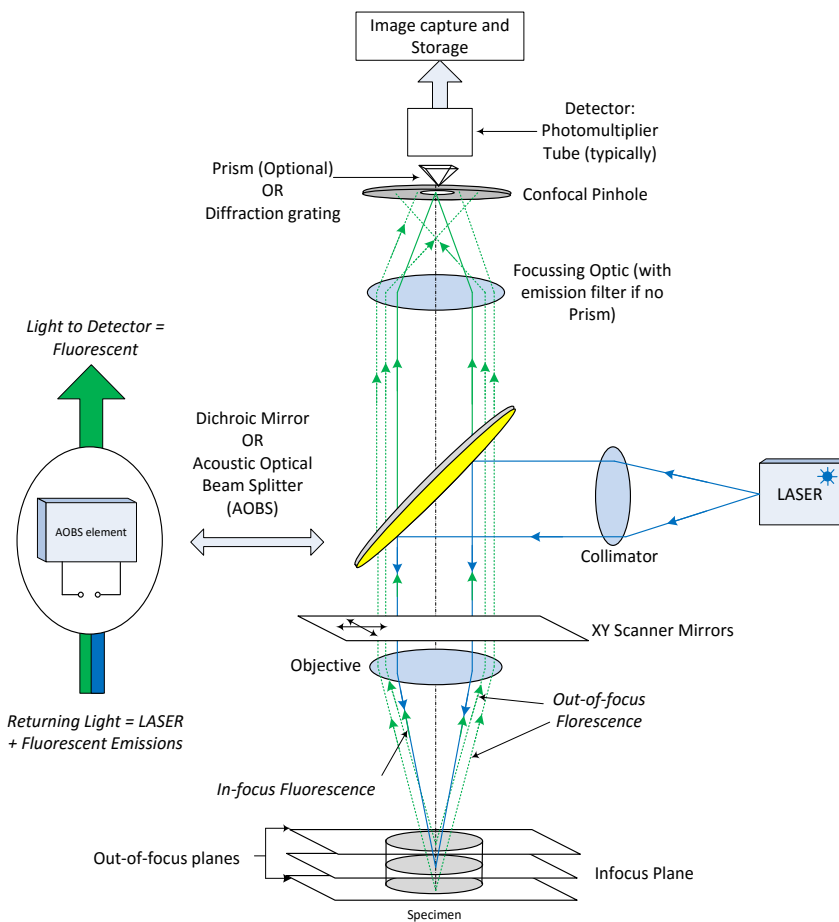


Figure 3.1: Modern Confocal Fluorescence Microscope (CFM) architecture showing how out-of-focus light is eliminated through the use of a confocal pinhole.

A dichroic mirror or an acoustic-optical beam splitter (AOBS) is used to reflect the laser light onto the sample which is marked with fluorophores. AOBSs are essentially AOTFs used spectrally to combine or separate laser light at a given wavelength from all other light). This causes the fluorophores to isotropically fluoresce light at a wavelength typically 20-100nm longer than the excitation wavelength (Stokes shift). This fluorescent light is then refocussed by the objective lens onto the dichroic mirror or AOBM which transmits the light onto the confocal pinhole.

The pinhole is the heart of a confocal microscope. As illustrated the undesired emissions (i.e. those from planes above and below the plane in focus), being non-confocal with the pinhole (with a small diameter) are not picked up by the detector. This effectively excludes extraneous light thereby sharpening the image of the point being sampled [4]. By means of a scanning mechanism many points on the object plane can be processed in a similar fashion. Scanning is usually achieved using plane mirrors whose tilt is controlled rapidly and precisely by galvanometers. Although early confocal microscope could only achieve rectangular scans (in a raster scan format), today's systems can scan arbitrarily shaped areas which is useful in Fluorescence Recovery after Photobleaching (FRAP) experiments; in FRAP a high intensity illumination spot can be precisely translated onto the desired (based on some biological criteria) region of interest on the specimen. Some modern confocal microscopes also incorporate band-pass emission filtering or spectral detection (per pixel basis) to enable multi-spectral imaging. For this purpose additional components such as prisms or diffraction gratings are included between the confocal pinhole and the detector.

3.2 Advantages of CFM

Confocal fluorescence microscopy provides several advantages over widefield fluorescence microscopy but the two most notable ones are: (i) elimination of out-of-focus light, and (ii) ability to take optical slices of sample in situ [1]. These and some of the other advantages are discussed below.

3.2.1 Non-invasive optical sectioning – With CFM, images of very thin optical sections (500 nm to 1 μm) of a thick specimens (>50 μm) can be serially produced without physically slicing the specimen. By assimilating images from different optical slices a very detailed picture of the sample can be reconstructed using volume rendering deconvolution methods [5-7] revealing hidden structures and features which are otherwise invisible to conventional microscopes [1]. Figure 3.2 [8] illustrates this 3D imaging process based on optical slicing.

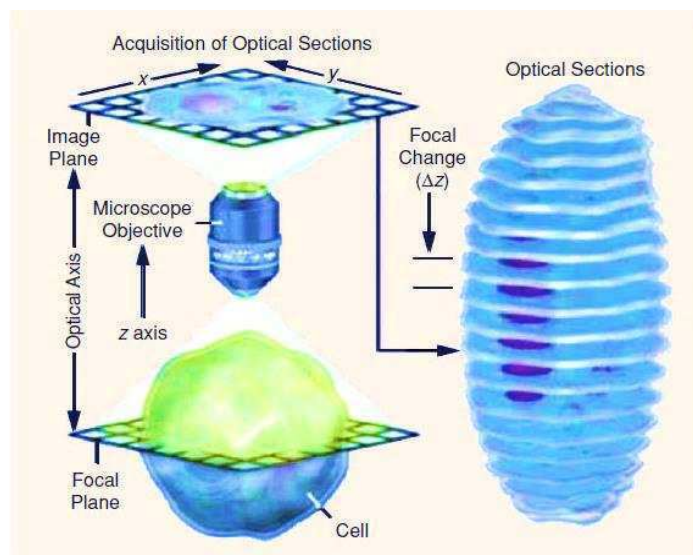


Figure 3.2: Process of 3D reconstruction using Confocal microscopy. 2D images of the different sections of the cell shown are serially produced. They are later assimilated to construct the 3D image of the cell [8]. Today's CFM systems are equipped with 3D software packages that can even create videos using several time lapsed 3D reconstructed images; useful for live cell imaging.

3.2.2 Improved Resolution and contrast – By rejecting out-of-focus fluorescence both resolution and signal-to-noise ratio are improved over widefield techniques. While imaging multicellular samples, complex tissues, or multi-layered structures (all common in biological applications) via conventional widefield microscopy light entering the detector from the out of focus planes severely degrades the image quality [1].

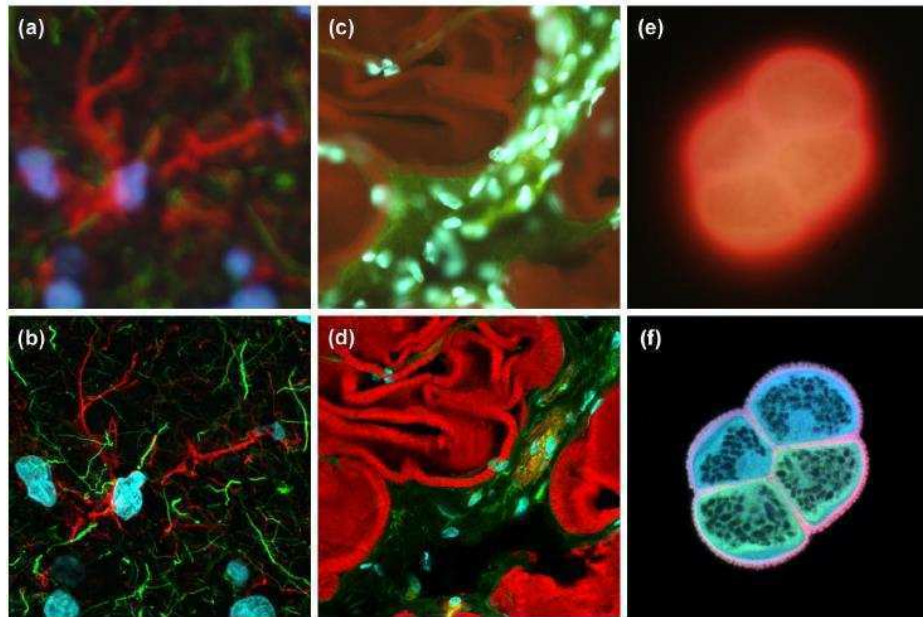


Figure 3.3: Comparing performance of conventional widefield imaging (a), (c) and (e) with confocal microscope imaging (b), (d) and (f) [4]

However, by the incorporation of a pinhole, CFM eliminates out-of-focus light thereby yielding information specific only to the focal plane (i.e. particular layer) referred to as an “optical slice”. Additionally CFM also rejects stray light from within the microscope itself that further improves image contrast and the signal-to-noise ratio along the focal plane [2]. Figure 3.3[4] illustrates the brilliance of CFM (bottom row) over conventional wide field microscopy (top row). When the pinhole is made vanishingly small, the PSF intensity in the in-focus plane for a circular aberration free lens can be approximated as,

$$I(v) = I_o \left| \frac{J_1(v)}{v} \right|^4, \quad (3.1)$$

where I_o is the intensity of the PSF at the centre of the infocus plane. The axial response to a uniform planar object can similarly be approximated as [9],

$$I(u) = I_o \left| \frac{\sin\left(\frac{u}{4}\right)}{\frac{u}{4}} \right|^4, \quad (3.2)$$

Where u and v are the normalised radial and axial optical units defined in equations 2.3 and 2.4 respectively. The lateral PSF and axial response of a CFM using equation 3.1 and 3.2 is shown in figures 3.4(a) and (b) respectively.

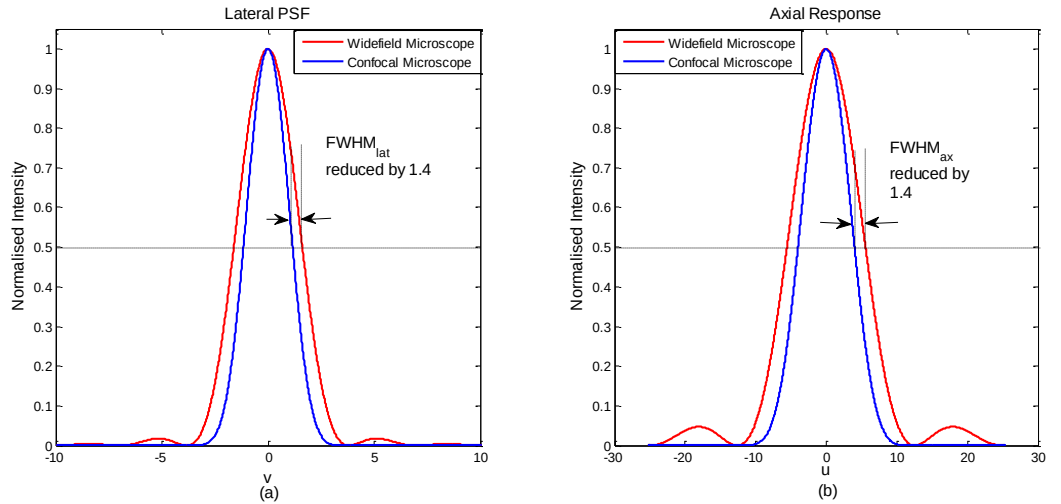


Figure 3.4: PSF of a widefield microscope vs PSF of a confocal microscope (very small confocal pinhole radius) seen as 1D cross section of (a) along x/y directions. (b) Axial (z-direction) response. FWHM_{lat} and FWHM_{ax} refer to the lateral and axial full-width-half-maximum widths respectively. The intensities have been normalised by the intensity at the centre of the PSF.

A resolution improvement by a factor of $\sqrt{2}$ (FWHM based) over a widefield microscope can be clearly seen. However, CFM in practice will not experience this precise resolution owing to lens aberrations (chiefly spherical and chromatic dispersion) that cause reduction as well as spread of illumination intensity over the image plane. This highlights the role of a good quality objective in determining the amount of light transmitted (signal strength),

image contrast and overall system resolution [10]. Contrast improvement can be demonstrated for a two point object (equal intensity peaks) separated by the Rayleigh distance of 3.86 normalised optical units, which is the distance at which a ~26% contrast is observed. Using a confocal microscope, for the same object separation, ~70% contrast can be achieved as shown in figure 3.5; almost 3 times the contrast.

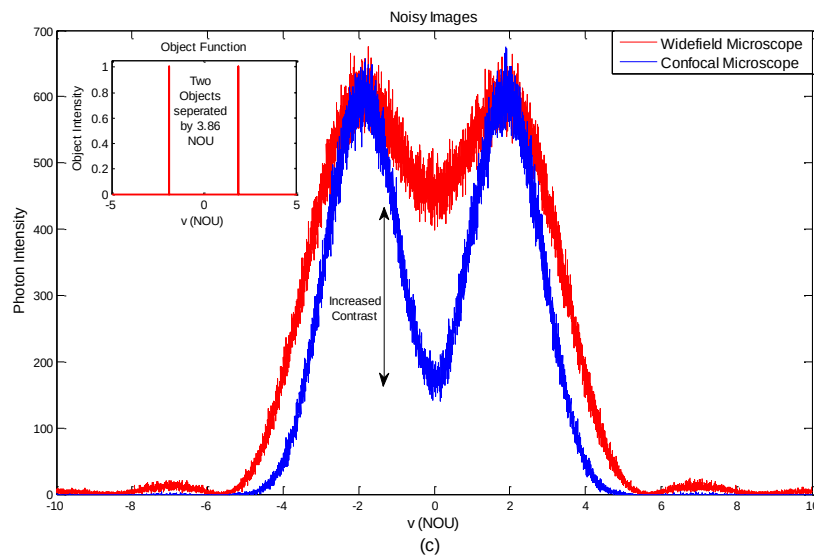


Figure 3.5: Simulated noisy widefield and confocal microscope images of a two point object (the two points are separated by the Rayleigh resolution limit for widefield microscope). The contrast improvement is clearly observable here.

3.2.3 Digitization - Sequential analogue image data gathered by the confocal microscope detector allows quantization of the continuous voltage signal into discrete output data[4]. Also quantitative measurements of the spatial fluorescence intensity can be obtained from this digital data either statically or as a function of time.

3.2.4 Electronic magnification - In CFM it is possible to electronically adjust the magnification (varying the region of laser scan) without the need to change objective lens. Termed as ‘zoom factor’ it is used to control the spatial resolution of the image by changing the sampling period of the illuminating

laser beam [11]. By increasing the zoom factor, the effective specimen area being scanned is reduced and the scanning rate is also simultaneously reduced, which results in an increased number of samples along a comparable length which in turn increases both the display magnification and image spatial resolution on the host computer [12]. When low NA – low magnification apertures are used, zoom factor allows the optical resolution of the microscope to match the digital image resolution [13, 14].

3.3 Limitations of CFM

3.3.1 SNR-resolution trade-off: The pinhole does a very good job of rejecting out of focus light and as shown in section 3.2.2 allows confocal systems to offer up to 1.4 times the resolution of widefield systems. This however comes at a price, namely the significant reduction in the amount of emitted (fluorescent) light that reaches the detector. Theoretically, the maximum resolution can be achieved when the pinhole is infinitesimally small. This in practice is obviously unacceptable as the system would detect almost no photons. As explained in chapter 1, for a shot-noise limited system such as CFM, the best achievable SNR is proportional to the square root of the number of detected photons. So a small pinhole would result in a poor SNR. This imposes a trade-off between SNR and resolution; attempting to improve one will degrade the other. Figures 3.6-3.9 illustrate this trade-off through computer generated images. Increasing the pinhole radius increases the total received signal thus improving the signal to noise ratio but degrades the lateral resolution.

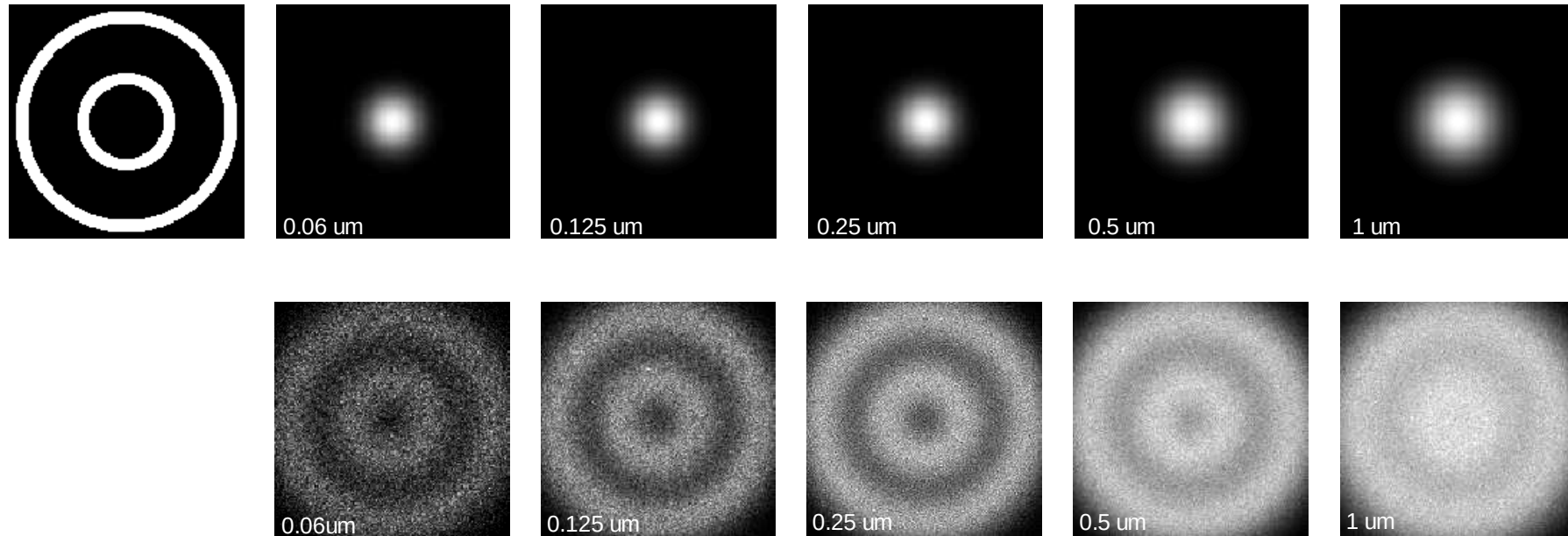


Figure 3.6: Simulated CFM images (bottom row) of a sample object (top left) at different pinhole radii. The sample planar object consists of two concentric rings each of $1\mu\text{m}$ width and separated by $4\mu\text{m}$. Here $\text{NA} = 0.5$ and $\lambda = 500\text{nm}$. The confocal PSF responses at different pinhole radii are also shown for reference (top row). Each image has been normalised by its own maximum intensity for ease of viewing. Relative intensities at pinhole radii of $0.06\mu\text{m}$, $0.125\mu\text{m}$, $0.25\mu\text{m}$, $0.5\mu\text{m}$ and $1\mu\text{m}$ are approximately 2%, 8%, 30%, 73% and 100% respectively.

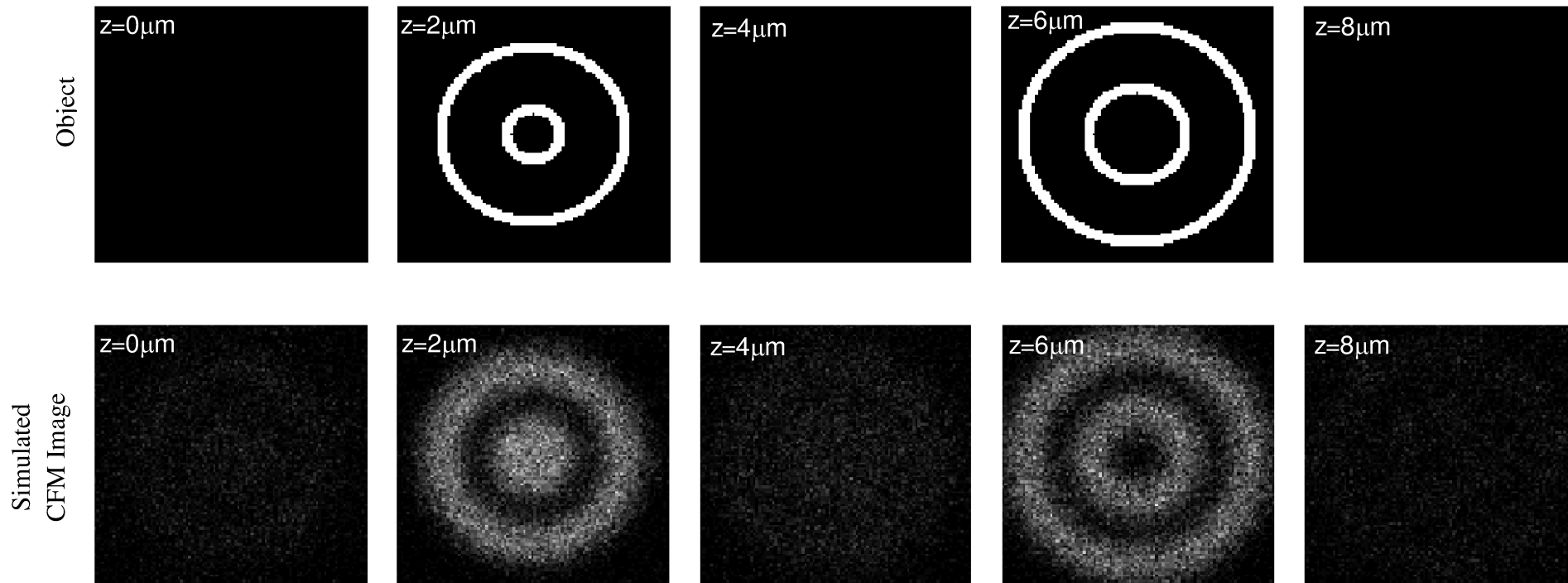


Figure 3.7: Simulated CFM images (bottom row) of a sample object (top row) as the object is moved relative to the focal plane; shown here for pinhole radius of very small pinhole radius ($0.06\mu\text{m}$). The simulated sample contains annular ring structures in the $z = 2\mu\text{m}$ and $z = 6\mu\text{m}$ planes as shown (but no structures in other planes). Each ring is of $1\mu\text{m}$ width and the concentric rings shown are separated from one another by $4\mu\text{m}$. Here $\text{NA} = 0.5$ and $\lambda = 500\text{ nm}$.

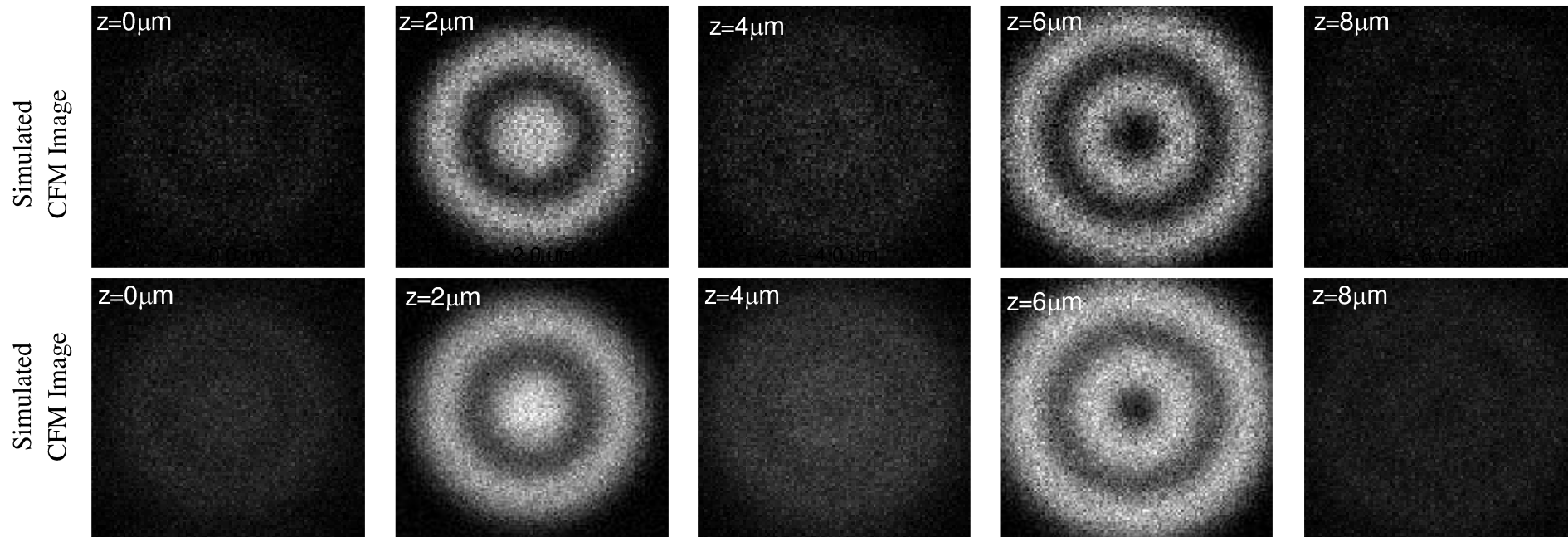


Figure 3.8: Simulated CFM images of a sample object (top row in figure 3.7) as the object is moved relative to the focal plane; shown here for pinhole radii of $0.125 \mu\text{m}$ (top row) and $0.25 \mu\text{m}$ (bottom row). Here $\text{NA} = 0.5$ and $\lambda = 500 \text{ nm}$.

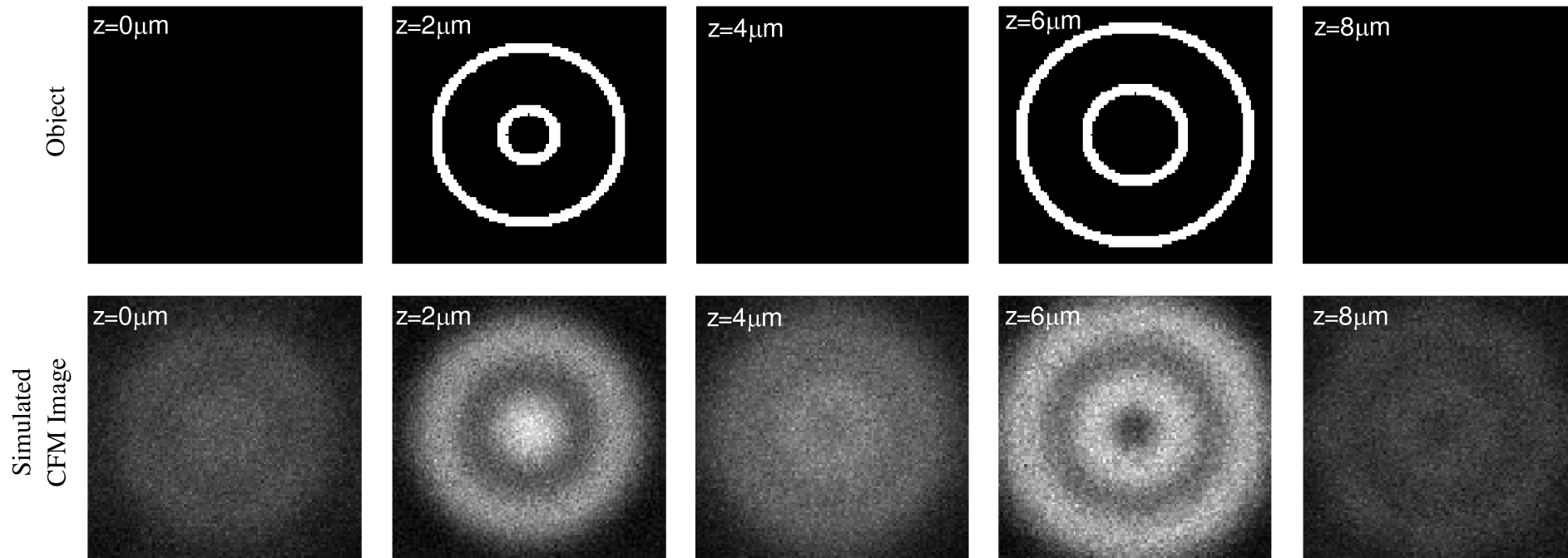


Figure 3.9: Simulated CFM images (bottom row) of a sample object (top row) as the object is moved relative to the focal plane; shown here for a large pinhole radius ($1\text{ }\mu\text{m}$). Here $\text{NA} = 0.5$ and $\lambda = 0.5\text{ }\mu\text{m}$.

3.3.2 Limited excitation wavelengths: In confocal microscopy, lasers are limited to a small number of excitation wavelengths (called laser lines) which are located in very narrow bands; excitations in the ultraviolet region can be difficult to produce. Some most commonly used laser excitations are: 458, 476, 488, 514, 543, and 633 nm. An implication of these limited laser lines is a limitation on the type of fluorophore that can be used. For example the Texas Red/Alexa594 fluorophore should not be used, since its excitation maximum (594nm) is far away from both the 543 nm and 633 nm laser lines. Widefield microscopes on the other hand that usually use mercury or xenon based lamps that are capable of excitation wavelengths in UV, visible as well as near IR spectra. This said however, staining specimens with fluorophores that fluoresce in the visible region are generally preferred, for they can be observed directly under the microscope with the naked eye. As resolution is inversely proportional to wavelength, one might consider selecting fluorophores that fluoresce at the blue region. Unfortunately at lower wavelengths autofluorescence and autoabsorption increases which reduces the SNR [15]. Furthermore, operating in the red region of the spectra is not generally preferred due to poor detector sensitivity commonly experienced in this region. For the above reasons, fluorophores emitting at green wavelengths (e.g, Alexa 568) are most preferred.

3.3.3 Other issues

- The focussing of a high intensity laser beam can be harmful to the specimen. It can also cause photobleaching and photodamage to the fluorophores.

- CFM systems are generally more expensive than widefield systems. However, cost shared microscope systems can be used by several departments of an imaging facility to reduce per user cost [9].
- The point scanning nature of the laser light in CFM reduces the data acquisition speed which makes CFM unsuited for many real time imaging applications.

3.4 Practical considerations in CFM

Before proceeding to evaluate the performance of a CFM, a discussion on some important design considerations is necessary. These are discussed next.

3.4.1 Photon efficiency in CFM

Photon efficiency defines the fraction of photons generated by the interaction of the laser beam with the specimen that are actually recorded to form the final image. Photon inefficiencies can be linked to several loss mechanisms. One such mechanism is introduced by backscattered light (BSL). This is the light reflected back to the laser source (via the dichroic mirror) from refractive index changes experienced by the scanning laser beam as it interacts with specimen. Pawley [16] has shown however that a BSL image can be combined with the fluorescent image to yield more information on the specimen's optical inhomogeneities without risking photodamage. A second mechanism is the excess light impinged onto the sample during the line retracing process (inherent to the raster-scan method). Fortunately, this loss can be overcome using acousto-optical deflectors (AODs) which essentially gate the light source during retrace. Other loss mechanisms include absorption and scattering of the laser light in the medium coupling the specimen to the

objective, in the objective itself or by scanning optics. Even an objective of NA of 1.4 collects only 30% of the isotropically scattered fluorescence. Depending on the wavelength of fluorescent light and objective NA, 20-95% of light collected is transmitted by standard objectives onto the following DM and descanning optics. These in turn transmit a relatively small 10-20% of the input light onto the photodetector. Using a small pinhole diameter, as little as 1% of the fluorescent light may be actually captured by the photodetectors. E.g., for a 1 mW laser composed of 10^{15} photons per second (512 x 512 scan per second) only about 10^7 photons will actually be detected viz., 100 photons/pixel/mW. Low quantum efficiency (QE) and extrinsic noise of the photodetector, misaligned optics (resulting from improper pinhole placement or orientation) and digitization errors (resulting from under or over sampling of the detector plane as discussed in chapter 2) of the photodetector output can further increase the photon inefficiency of a CFM.

3.4.2 Detector choice

CFMs usually employ photomultiplier tubes (PMTs) (see fig 3.10(c)), avalanche photodetectors (APDs) (see fig 3.10(d)) or solid state charge couple device (CCDs) (see fig 3.10b)) cameras as detectors. The following section discusses the suitability of using the three detector types in CFM.

PMTs: The most commonly used detector in CFM is the PMT. Most of the PMTs offer a QE as high as 30%. But, QE performance is wavelength dependent (see fig 3.10(a)) and a multialkali coated photocathode based PMT for example offers only 15% QE at the commonly used fluorescence microscopy wavelength of 565 nm. Using mechanisms that optically enhance

the light striking the end plane of the Photocathode tube the QE can be increased by 60% at 520 nm and by 180% in the red region of 690nm. Also newer photocathodes such as Gallium Arsenide (GaAs) and GaAs-Phosphide (GaAsP) exhibit considerably higher QE in the red region. GaAsP PMTs have in fact become a standard for CFM systems over the last few years [17].

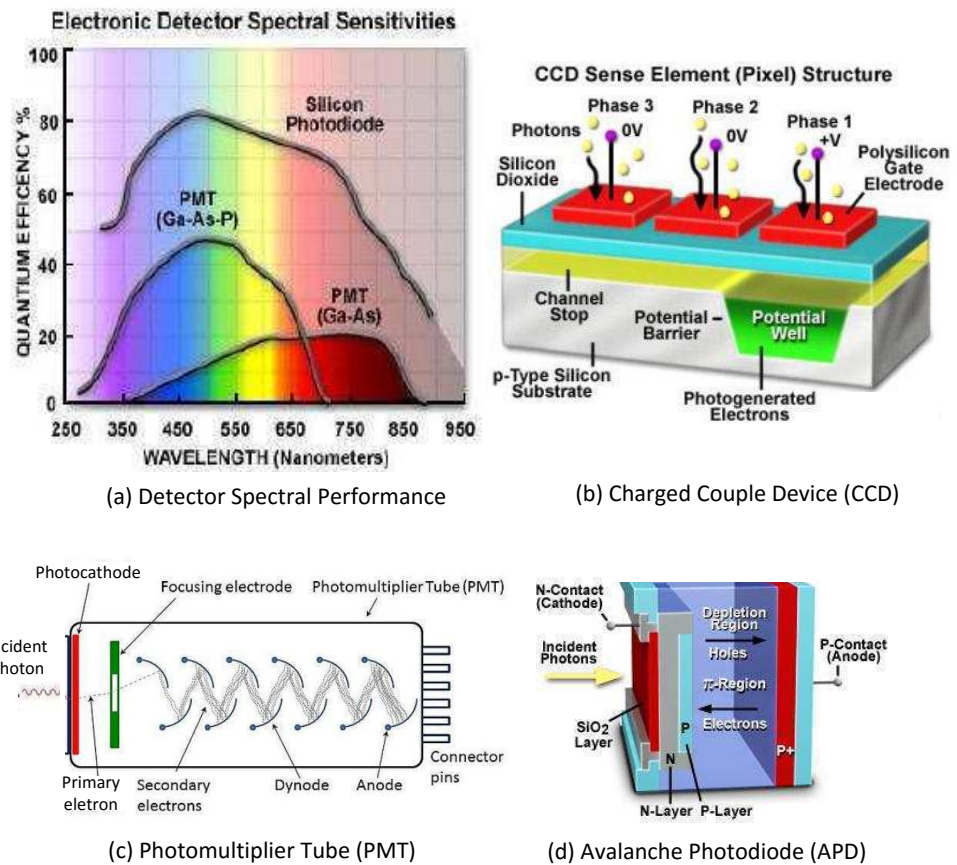


Figure 3.10: (a) Quantum efficiencies are wavelength dependent. CCDs exhibit a broad spectral characteristic with higher QE than the narrow band PMTs. (b) In a single CCD element, charge is built up in a potential well (upon photons inducing photoelectron generation in the p-n junction) and then transferred to a serial register at the bottom of the corresponding pixel column of the CCD array. All the pixels are read out in a “bucket brigade” method for digitization and image generation. (c) In a PMT, photoelectric conversion occurs at the photocathode, after which dynodes provide amplification through secondary emissions. The photoelectrons are then accelerated towards the anode by an electric field. (d) In a APD, photons entering the diode diffuse through the SiO_2 layer and then through the n and p layers before entering the depletion region where they excite free electrons and holes, which then migrate to the cathode and anode, respectively. When a semiconductor diode has a reverse bias (voltage) applied and the crystal junction between the p and n layers is illuminated, then a current will flow in proportion to the number of photons incident upon the junction.

PMTs normally produce low dark currents (0.1 counts/second) but in warmer confines of a microscope can produce considerable dark current noise (several 1000 counts/second). Thus PMTs are normally maintained at low temperatures. Dark current is also dependent on the size of the photocathodes and so smaller photocathode diameters are used in CFM systems. Another point to consider is that PMTs give rise to multiplicative noise (which reduces QE) due to the statistical nature in the gain at the early stages of photon multiplication. Fortunately, multiplicative noise can be reduced using new hybrid PMTs which achieve this by inducing a very high gain in the very first photon multiplication stage. PMTs being single channel devices, care must be taken to align them with the detection pinhole in CFM.

APDs: Avalanche photodiodes are similar in design to p-i-n photodiodes but have a relatively thin depletion region which results in a very highly localised electrical field in the narrow depletion region. APDs provide gain by the generation of electron-hole pairs which result in an “avalanche” of electrons in the substrate. Their operation requires very high reverse bias voltages (100V - several 1000s of volts) such that in general a larger reverse bias results in a higher APD gain [18]. Although APDs are more compact and durable than PMTs, capable of moderate (500-1000 in non-Geiger-mode) to high (up to 10^8 in Geiger mode) gain and offer QEs of up to 90%, they exhibit high levels of dark current noise which substantially raises the noise floor (resulting in lower SNR). APDs also have smaller active area than PMTs which is advantageous but also means that it can be difficult to sustain focus onto the small region; additional optical elements are thus required which increase system complexity. They are preferred for imaging applications beyond 700 nm (edge

of the visible spectrum) because their QEs are higher at longer wavelengths [19]. APDs offer higher dynamic range (up to 3 orders of magnitude) than PMTs making them better suited for low light CFM applications [20]

CCDs: Offering QEs as high as 80%, CCDs appear to offer a better alternative to PMTs for low-light imaging. As individual photons are recorded as an identical quantity of current, CCDs do not suffer from multiplicative noise. However to keep dark current and readout noise levels low, they need to be cooled and be read out up to 4 times slower than the conventional CFM scan speed (viz., 0.4 Mpixels/s) [13]. Because of this cooled CCDs are better suited for slow-scan systems. The introduction of Electron multiplier CCDs (EM-CCDs) is a significant development. EM-CCDs produce average gains of up to 1000 thus lifting the signal well above the read-out noise floor. However it suffers from multiplicative noise effects as in a PMT and requires considerable cooling. Another point to be taken into consideration is that by geometry CCD arrays do not have detectors over the entire surface as some real estate is assigned to electronics. This means a considerable portion of the array isn't available for photon detection. Another possible detector choice is the Complimentary Metal-Oxide Semiconductor (CMOS) camera. CMOS detectors like CCDs use pixel sensors but are much faster as they complete the digitization at each pixel. It also consumes up to 100 times less power than CCDs. They are however not yet suited for low-light imaging, as in CFM, due to higher levels of amplifier noise, lower QE and smaller dynamic range.

3.4.3 Imaging with high NA

To increase NA beyond unity, high refractive index immersions (between sample and cover slip) are commonly used. Although these higher NA systems

generally provide increased resolution, they reduce the depth of field which makes optical sectioning of thick specimens more challenging. Also overcoming chromatic aberrations becomes more challenging in these systems. Equation 3.1 is based on the paraxial approximation (PA) which is adequate for low and moderate NA systems ($\sin \alpha < 0.8$ or $NA < 1.25$ with an oil immersion objective [21, 22]). All analysis in this thesis assumes PA and thus applies to moderate NA systems; Pawley [13] notes that PSF modelling for high NA systems using PA would result in: no alteration in the ‘Gaussian like’ shape of the PSF, an overestimation of the PSF widths but only by a small amount and non-inclusion of the marginally raised PSF sidelobes that are observed [13]. This ‘overestimation’ or scaling of the PSF made by conventional PA approaches has been by not taking into account the vectorial character of the light. However, different vector-based theories [23-28] yield significantly different scaling values and the number of experimental investigations remains limited to this day [26, 29, 30].

3.5 Impact of pinhole on CFM performance

From the description of the confocal imaging process in section 3.2, it is clear that the pinhole geometry plays an important role in shaping the PSF and thus deciding the resolution of a CFM system. Equation 3.1 represents the PSF of a CFM system (assuming an aberration free objective and paraxial approximation) when the pinhole is made infinitesimally small. In practice a finite sized pinhole is always used to sacrifice resolution for better signal-to-noise ratio. The analytical expression for the confocal PSF using a finite sized circular pinhole will be now be derived.

CFM imaging with a finite sized pinhole

Consider a 3D object with fluorophore concentration $O(v, u)$, illuminated by a lens (point spread function PSF_1), being imaged onto the detection plane of a CFM system (schematic shown in figure 3.11) using another lens (point spread function PSF_2). A circular pinhole with transmittance $Pin_{circ}(v, v_d)$ defined mathematically as,

$$Pin_{circ}(v, v_d) = \begin{cases} 1 & \text{if } v \leq v_d \\ 0 & \text{otherwise} \end{cases}, \quad (3.3)$$

is placed just before a detector – e.g. a photomultiplier tube (PMT). At a given scan position $S(v_s, u_s)$, the mean number of photons arriving at the pinhole is given by the convolution of PSF_1 weighted by the object fluorescence concentration $O(v, u)$ and PSF_2 ,

$$n(v_s, u_s, v, u) = \int_{v'} \int_{u'} O(v' - v_s, u' - u_s) PSF_1(u', v') PSF_2(u - u', v - v') dv' du' \quad (3.4)$$

Here v and u are optical radial and optical axial coordinates related to object radial displacement r and object defocus z by equations (3.5) and (3.6) respectively [31].

$$v = krsin(\alpha), \quad (3.5)$$

$$u = kzsin^2\left(\frac{\alpha}{2}\right), \quad (3.6)$$

where α is the lens semi-aperture angle, λ is the illumination wavelength and $k = 2\pi\eta/\lambda$ (η is the refractive index). Here $\sin \alpha \approx a/f$ is the numerical aperture of either lens where a is the pupil radius, f is the focal length

(assumed equal for both lenses under unity magnification). This is a good approximation if $f \gg a \gg \lambda$ and $a^2/\lambda f \gg 1$ [32].

The mean number of photons detected by the PMT is then the integral of the product of $n(v_s, u_s, v, 0)$ in the focal plane ($u = 0$) with the pinhole defined in equation 3.3,

$$I(v_s, u_s) = \int_v Pin_{circ}(v, v_d) \int_{v'} \int_{u'} O(v' - v_s, u' - u_s) PSF_1(u', v') PSF_2(-u', v - v') dv' du' dv . \quad (3.7)$$

Re-arranging the terms,

$$I(v_s, u_s, v_d) = \int_{v'} \int_{u'} O(v' - v_s, u' - u_s) PSF_1(u', v') \int_v Pin_{circ}(v, v_d) PSF_2(-u', v - v') dv' du' dv \quad (3.8)$$

The terms under the v integral represents a 2D convolution ($\otimes$) between the pinhole and the detection lens PSF. Equation 3.4 can thus be re-written as,

$$I(v_s, u_s, v_d) = O(v, u) \otimes (PSF_1(v, u) \times (PSF_2(v, u) \otimes Pin_{circ}(v, v_d))) \quad (3.9)$$

For a point object located at the centre of the focal plane, equation 3.9 represents the PSF of the CFM system, written as [33],

$$PSF_{conf}(v, u, v_d) = PSF_1(v, u) (PSF_2(v, u) \otimes Pin_{circ}(v, v_d)). \quad (3.10)$$

It is assumed throughout that the excitation and emission wavelengths are equal (i.e zero Stokes shift).

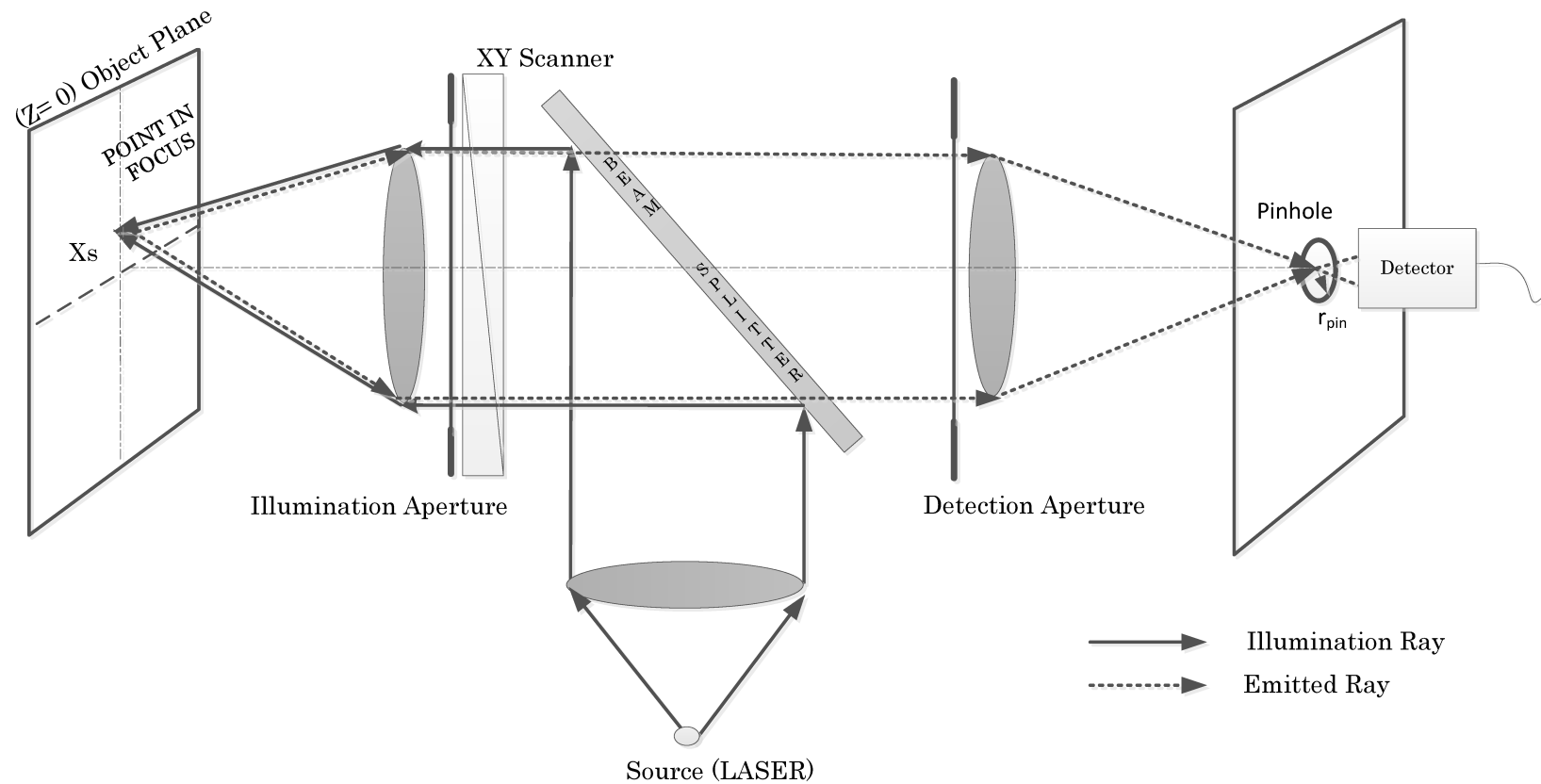


Figure 3.11: Schematic of a CFM system showing how fluorescence from a single scan point is focussed by the optics onto a circular pinhole aperture. The diffracted light that falls within the pinhole aperture is integrated by the detector, digitised and converted to a set of gray levels by a computer.

The lateral resolution may be determined by computing the full-width half maximum (FWHM) of the PSF along the lateral direction (see figure 3.12(b)). The axial resolution is computed by measuring the FWHM of the response of the system to a planar object as it is moved in the axial direction as shown in figure 3.12(a).

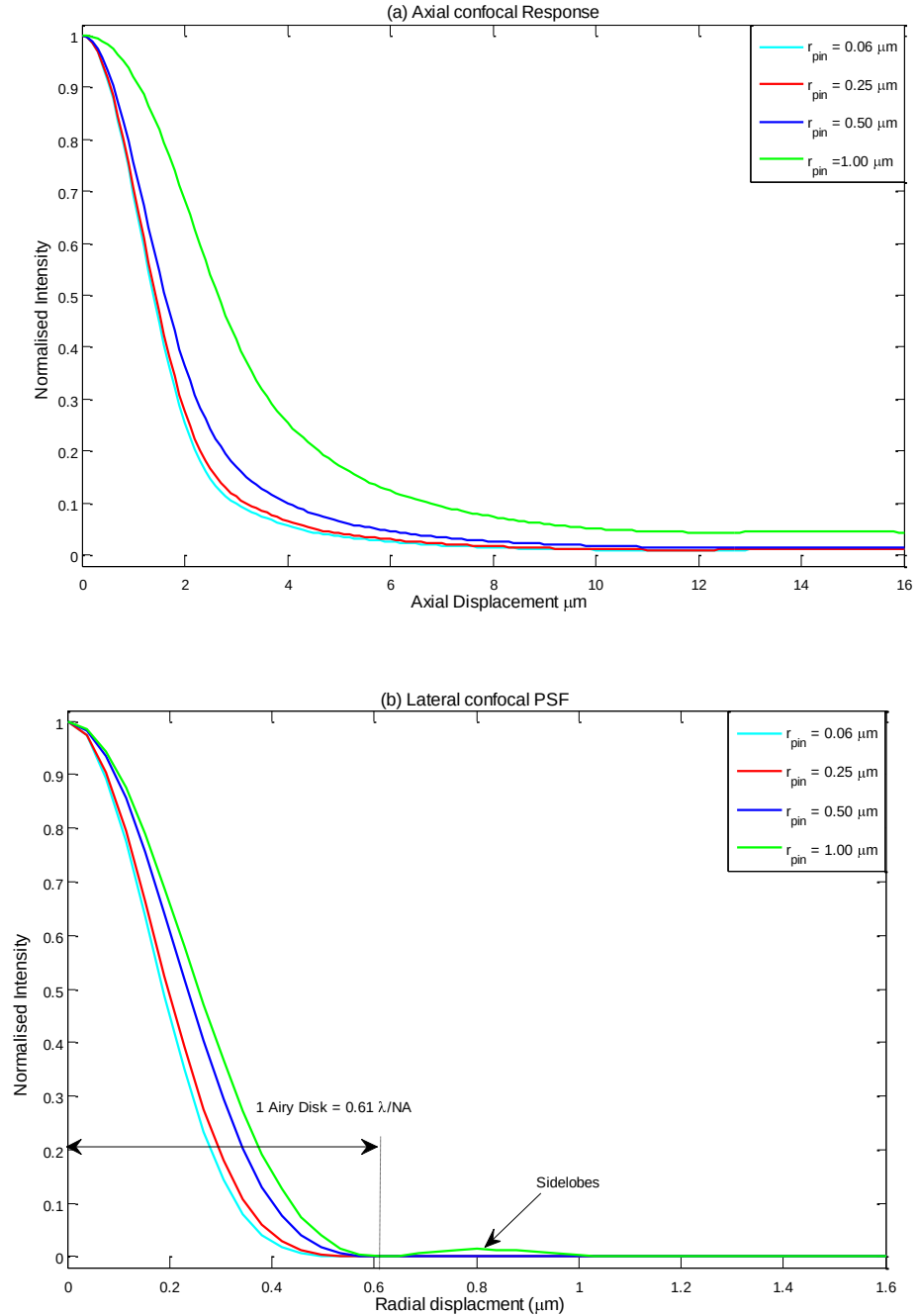


Figure 3.12: Effect of pinhole radius on (a) axial confocal response and (b) lateral confocal PSF. Increasing the pinhole radius not only widens the mainlobe and produces a larger detector signal (beneficial), it also introduces undesirable artefacts in form of sidelobes which add undesirable background noise. Here $\text{NA} = 0.5$ and $\lambda = 0.5 \mu\text{m}$.

Figure 3.14 (a) and (b) show the variation of lateral and axial resolution with pinhole radius. Here $NA = 0.5$ and $\lambda = 0.5 \mu m$. For pinhole radii $< 0.6 \mu m$ (viz., the radius of first Airy Disk of the lens PSF), the CFM system offers substantial improvement in resolution over a wide-field system, but reduces the amount of light falling onto the detector.

As explained in chapter 2, Poissonian statistics based shot noise is the dominant noise contributor in fluorescence microscopy which ultimately limits the performance of the CFM system (i.e. neglecting background from autofluorescence of the fluorophores and discounting detector dark noise). Thus, the mean and variance of the photon distribution at the image plane are equal and the SNR can be readily calculated as the ratio of the mean number of detected photons (determined at the image plane) to the standard deviation. A normalised version of the SNR for a planar uniform object of unity fluorescence in the focal plane reduces to the expression in equation (3.11), which has a normalised maximum value of unity for a large detector pinhole,

$$SNR_{limit} = \frac{\text{mean number of detected photons at image plane}}{\sqrt{\text{mean number of detected photons at image plane}}} \quad (3.11)$$

The mean number of detected photons can be calculated by integrating the photon distribution at the image plane image $I(v, u = 0)$ over the 2D image space. Thus equation 3.11 can be reduced to,

$$SNR_{limit}(v_d) = \sqrt{\int I(v, 0, v_d) v dv} \quad (3.12)$$

For uniform planar object of unity fluorescence (in the image plane) $I(v, 0) = PSF_{conf}(v, 0, v_d)$. Using equation 3.9, equation 3.12 can now be written as,

$$SNR_{limit}(v_d) = \sqrt{\int PSF_1(v, 0) \left(\int PSF_2(v', 0) Pin_{circ}(v - v', v_d) dv' \right) v dv} \quad (3.13)$$

For a given set of pupil apertures, the SNR limit of a CFM system increases with pinhole radius. As shown in the figure 3.13 the rate of increase of SNR is higher for smaller pinhole radii than at larger pinhole radii. However, the resolution vs pinhole characteristics (figure

3.14(a) and (b)) show that for smaller pinhole radii there is a gradual decrease in both lateral and axial resolution followed by a rapid degradation of the same at larger pinhole radii. Thus there exists an inverse non-linear relationship between SNR and resolution and it is interesting to examine their interdependence. Figures 3.14 (c) and (d) plot the $\text{SNR}_{\text{limit}}$ vs resolution characteristics along the lateral and axial directions respectively for a conventional circular pinhole. The curves characterise the SNR-resolution trade-off as the pinhole radius is varied.

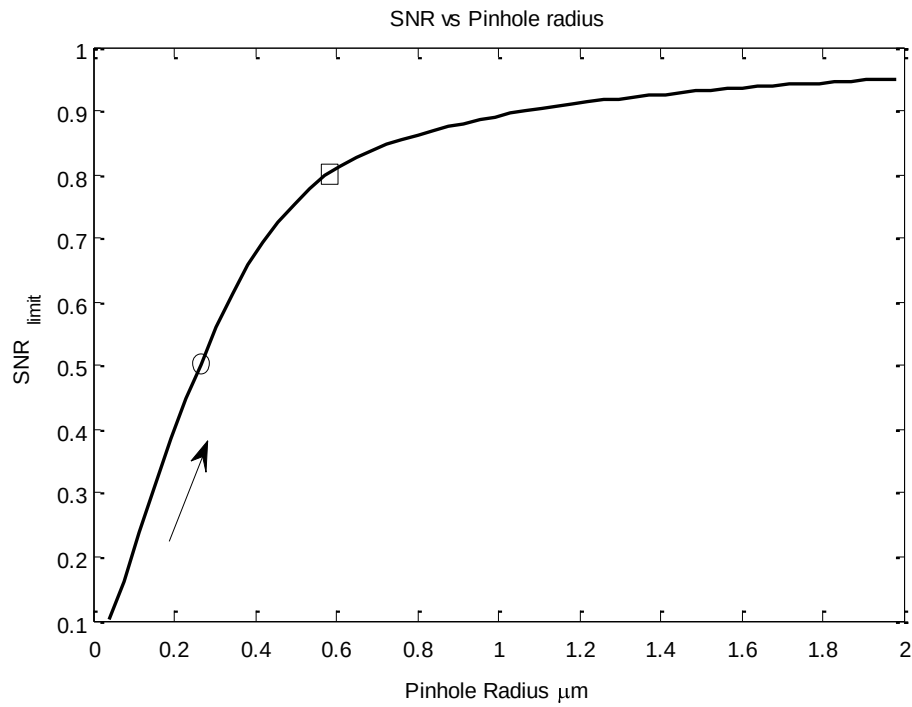


Figure 3.13: SNR as a function of pinhole radius in a CFM system. Here $\text{NA} = 0.5$ and $\lambda = 0.5 \mu\text{m}$. The $\circ$ and $\square$ indicate data points at pinhole radii of $0.25\mu\text{m}$ and $0.6\mu\text{m}$ respectively. The arrows indicate the direction of increasing pinhole radius.

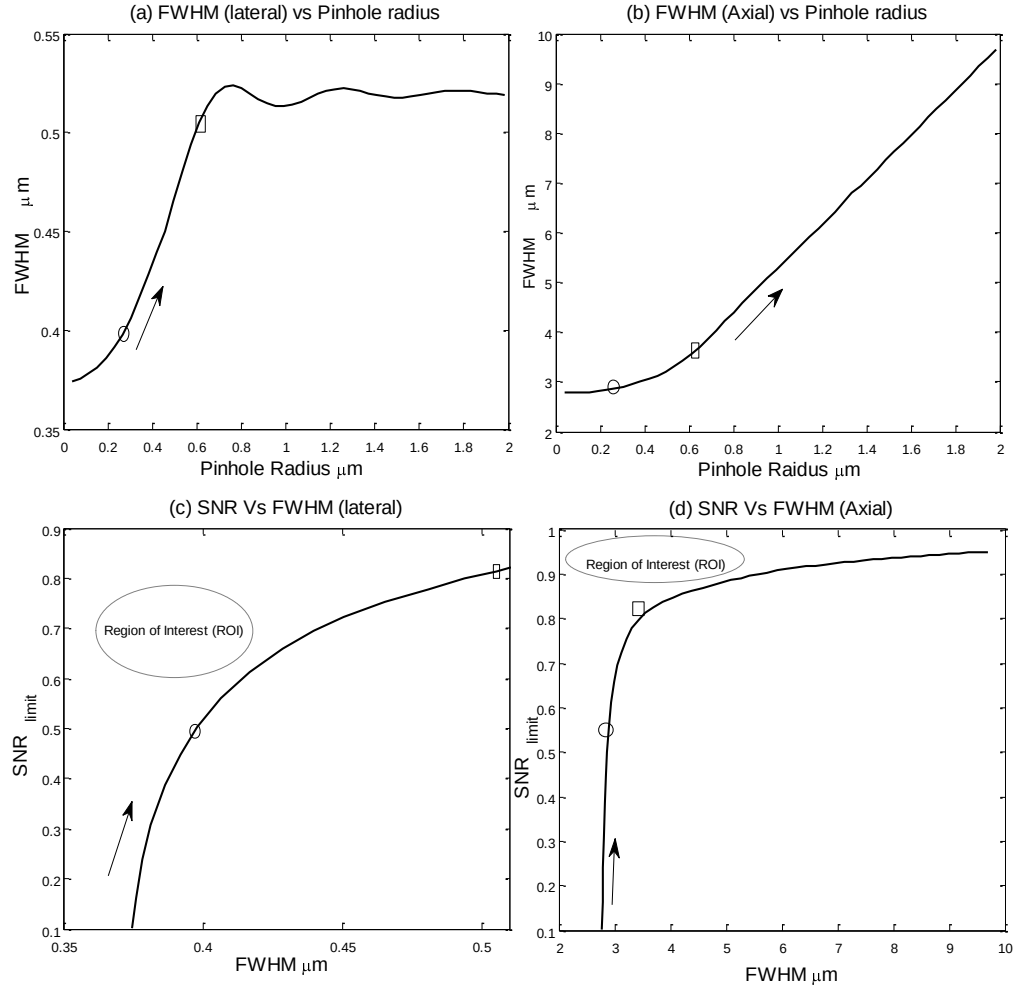


Figure 3.14: FWHM of the confocal PSF measured as function of pinhole radius in the (a) lateral and (b) axial directions; $NA = 0.5$ and $\lambda = 0.5 \mu\text{m}$. The $\circ$ and $\square$ indicate data points at pinhole radii of $0.25\mu\text{m}$ and $0.6\mu\text{m}$ respectively. (c) and (d) show the SNR-resolution trade-off along the lateral and axial directions respectively for a CFM system as the pinhole radius is varied. The arrows indicate the direction of increasing pinhole radius.

Generally, a pinhole radius < 1 Airy unit (which for a NA of 0.5 and $\lambda = 0.5 \mu\text{m}$ is $0.61 \mu\text{m}$) is used in CFM systems as almost 80% of the total PSF intensity is confined within this radius. As can be seen from figure 3.13 increasing the pinhole radius beyond $0.61\mu\text{m}$ results in no significant SNR gain. At small pinhole radii, the reduction in PSF intensity is accompanied by reduction in the PSF width which improves resolution in both directions (see figure 3.12). It is thus clear that the pinhole geometry plays an important role in controlling the resolution and SNR of a CFM system. It would be desirable

to employ a pinhole that would enable achieving performance in the ‘Region of Interest (ROI)’ as marked in figure 3.14. Operation in the ROI might be achieved by designing pinhole geometries that, for given pinhole size, offer better resolution at a given SNR, a better SNR at a given resolution or a better SNR as well as resolution (most preferred).

The next chapter will explore alternate pinhole geometries that will enhance both the SNR and resolution of a CFM system.

3.6 Chapter Summary

- Inherently a CFM system sacrifices signal to noise ratio to achieve its $\sqrt{2}$ resolution improvement over conventional widefield systems.
- The ability of the CFM to non-invasively examine biological specimens with superior resolution and contrast make them an indispensable tool in modern bio-imaging.
- The SNR-resolution trade-off sets the fundamental bounds on the performance of a CFM. As shown, this can be studied by analysing trade-off characteristics curves.
- Practical CFM design requires careful consideration in the choice of optical components. Although many modern CFM systems employ CCD detectors for their high quantum efficiency, PMTs continue to be used for several high speed confocal applications such as in FLIM and FRET.
- One of the key components is the detection pinhole; its geometry is responsible for setting the operating point on the SNR-resolution trade-off curve.

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CHAPTER 4

Enhancing CFM performance using alternative pinhole geometries and pixel array detectors

This chapter will explore the use of alternative pinhole geometries in conjunction with pixel array detectors to realise CFM performance in the trade-off characteristic's "regions of interest" (ROI). These regions, as described in the last chapter, correspond to desirable CFM operation points; controlled by the pinhole size and shape. To realise operation in the ROI, it must be shown that better SNR at a given resolution or vice versa can be obtained. Conventional CFM systems employ a small pinhole – circular shaped apertures which simply allow light within its defined pinhole radius to strike the following detector for signal generation. An alternative approach would be to replace the pinhole by a pixel array, the output of each pixel electronically weighted to emulate any pinhole geometry. Using new pinhole geometries, through computer simulation results, it will be shown that this detection scheme offers a better SNR-resolution trade-off in comparison to a conventional CFM system i.e. operation in the ROI will be demonstrated. The following section will describe the CFM imaging operation using pixel array detectors.

4.1 CFM imaging with pixel array detectors

Consider the same CFM system setup shown in figure 3.11, the detection side of which has been shown in figure 4.1(a), except now the pinhole and the point detector is replaced by an array of N pixel detectors as shown in figure

4.1(b). The output of each pixel is weighted by a certain value T_i and can be electronically controlled.

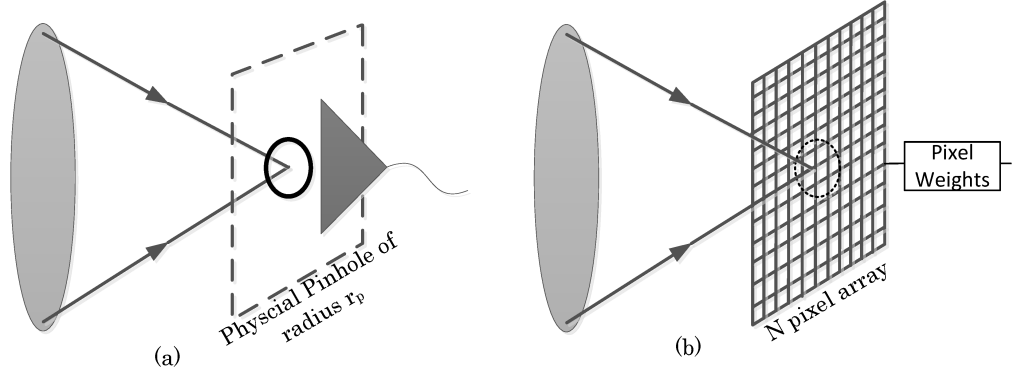


Figure 4.1: Detection arrangement in a (a) conventional pinhole-point detector based CFM system but where the pinhole may have a variable transmission coefficient and (b) pixel array based CFM system.

Under this scheme the detected signal S would be given by the summation of the product of the number of photons n_i arriving at pixel i and the pixel weight T_i ,

$$S = \sum_{i=1}^N n_i T_i. \quad (4.1)$$

For a circularly symmetric set of pixel weights the pixel array detector pinhole can be defined as,

$$Pin_{pix}(v_l, v_d) = \begin{cases} T_i & \text{if } v_l \leq v_d \\ 0 & \text{otherwise} \end{cases}. \quad (4.2)$$

where v_l represents the radial displacement (in optical units) in the detector plane and v_d the pinhole radius. The Poissonian shot noise (signal standard deviation) contributed by each pixel detector σ_i is then given by,

$$\sigma_i = \sqrt{n_i T_i}. \quad (4.3)$$

Now the total signal variance σ_S^2 is calculated by summing up the variances from each pixel,

$$\sigma_S^2 = \sum_{i=1}^N n_i T_i^2. \quad (4.4)$$

Assuming very weak autofluorescence, zero dark current noise and a shot noise limited detection, the SNR of the pixel array detector based CFM system would be given by dividing equation 4.1 by the square root of equation 4.4,

$$SNR_{pixel} = \frac{\sum_{i=1}^N n_i T_i}{\sqrt{\sum_{i=1}^N n_i T_i^2}}. \quad (4.5)$$

Now, if the number of pixels is made sufficiently large ($N \geq$ object scan points), then for a uniform planar object of unity fluorescence (in the focal plane) the mean number of photons arriving at the pixel array is given by the convolution of the intensity point spread functions of the two conjugate lenses,

$$n(v_I) = PSF_1(v_I, 0) \otimes PSF_2(v_I, 0). \quad (4.6)$$

Now, the numerator and denominator of equation 4.5 can now be evaluated as,

$$\sum_{i=1}^N n_i T_i \propto 2\pi \int n(v_I) Pin_{pix}(v_I, v_d) v_I dv_I. \quad (4.7)$$

$$\sum_{i=1}^N n_i T_i^2 \propto 2\pi \int n(v_I) Pin_{pixel}^2(v_I, v_d) v_I dv_I. \quad (4.8)$$

Substituting equation the above into equation 4.5,

$$SNR_{pixel}(v_d) = \frac{\int n(v_I) Pin_{pix}(v_I, v_d) v_I dv_I}{\sqrt{\int n(v_I) Pin_{pixel}^2(v_I, v_d) v_I dv_I}}. \quad (4.9)$$

Note that here a circular pinhole geometry implies that all pixels within a given pinhole radius v_d have unity weights i.e. $T_i = 1$ for $v \leq v_d$. For non-unity weights, SNR values calculated from equation 4.9 are generally greater than that derived from expression 3.13. In all the figures presented in this chapter, SNR_{pixel} is normalised such that it has a value of unity for a very large pinhole radius when circular pinhole geometry is used. It is noted that the pixel array detector based system offers the same SNR and resolution as

the conventional CFM systems for circular pinhole geometries. However by weighting the output of each detector element differently (i.e. within the pinhole radius $T_i \neq T_{i-1}$) different pinhole geometries can yield an improved SNR-resolution trade-off, as will be shown next.

4.2 Investigating new pinhole geometries

In principle, there exist an infinite number of pinhole geometries that could be investigated to find those that yield improved CFM performance. The following sections will investigate two pinhole families that will demonstrate the advantage of using different pinhole geometries in conjunction with pixel array detection in a CFM system. A more rigorous stochastic based investigation using a genetic algorithm will also be performed.

4.2.1 Triangular pinhole family: Consider a family of pinholes defined by the equation,

$$Pin_{pix}(v, v_d) = \begin{cases} \left(1 - \frac{v}{v_d}\right)^{ne} & \text{for } v \leq v_d \\ 0 & \text{otherwise} \end{cases} \quad (4.10)$$

which will be referred to hereon as a “triangular pinhole family”. The circular pinhole geometry used in conventional CFM (and defined by equation 3.3) can be approximated by equation 4.10 when shape parameter $ne \rightarrow 0$. Using equation 4.10, figure 4.2 plots different pinhole geometries that deviate from the standard circular pinhole profile ($ne = 0$) by varying ne between a small value of 1/32 and a large value of 32. Increasing ne decreases the pinhole area reducing the detector signal intensity (and hence the SNR) but also decreases the PSF mainlobe width along both the lateral as well as axial directions as shown in figure 4.3(a) and (b).

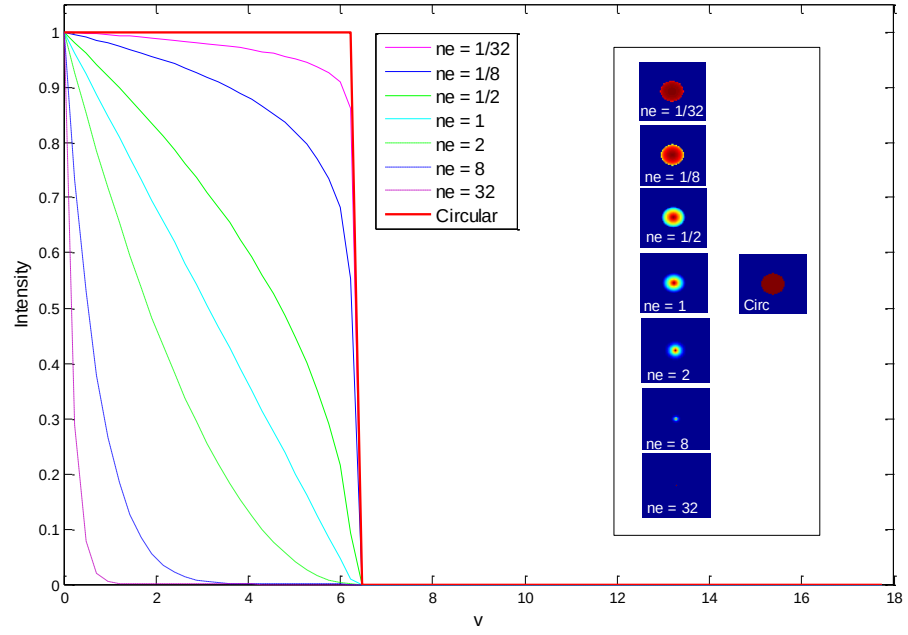


Figure 4.2: Effect of parameter ne (equation 4.10) on the pinhole shape. Here pinhole radius Here $v_d = 2\pi$. The intensities have been normalised by the peak intensity.

For example, at a pinhole radius of $0.6 \mu\text{m}$ and $ne = 4$, the pixel array detector based system offers 1.27 times lateral and 1.2 times axial resolution improvement over conventional circular pinhole-point detector based CFM systems. The pixel array detector based system suffers from a lower SNR than a circular pinhole-point detector based system but as can be observed in 4.3(c) and (d), for the same pinhole geometry (i.e. same value of ne) the pixel array detector based system suffers less SNR degradation when compared to conventional (variable pinhole) point detector based CFM system. For example at a pinhole radius of $0.6 \mu\text{m}$, the pixel array based system offers 1.45 times the SNR offered by conventional (variable pinhole) CFM system when $ne = 2$. Alternative pinhole geometries using pixel based detectors can thus yield a better SNR-resolution trade-off than conventional CFM systems. Figure 4.4 shows how using the new detection scheme, CFM performance can now be observed in the ROI.

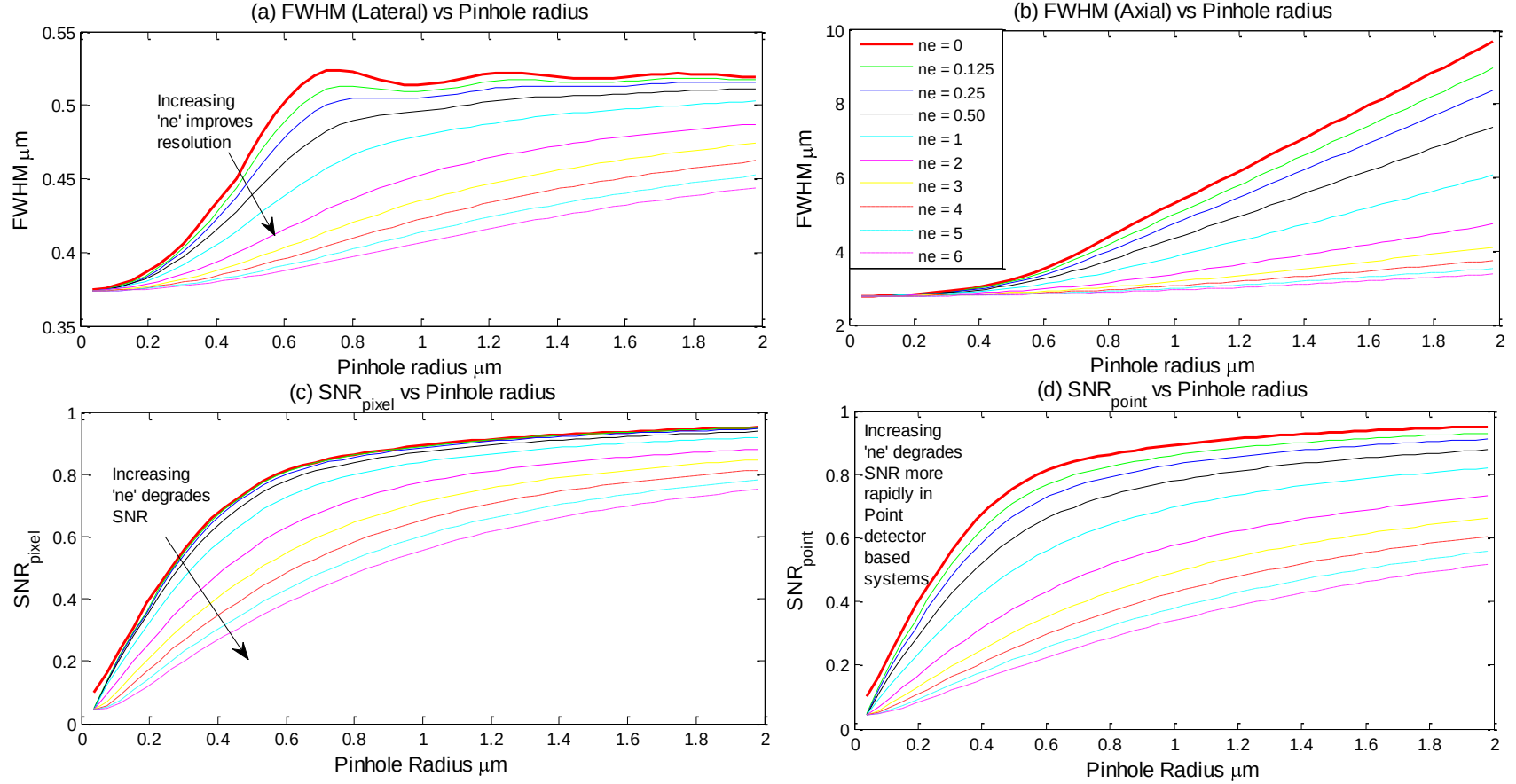


Figure 4.3: Effect of parameter ne (see equation 4.10) on FWHM of confocal PSF in the (a) lateral and (b) axial directions. Effect of parameter ne on the SNR of a (c) pixel array based detector and (d) point detector based CFM system. Here $NA = 0.5$ and $\lambda = 0.5\mu\text{m}$.

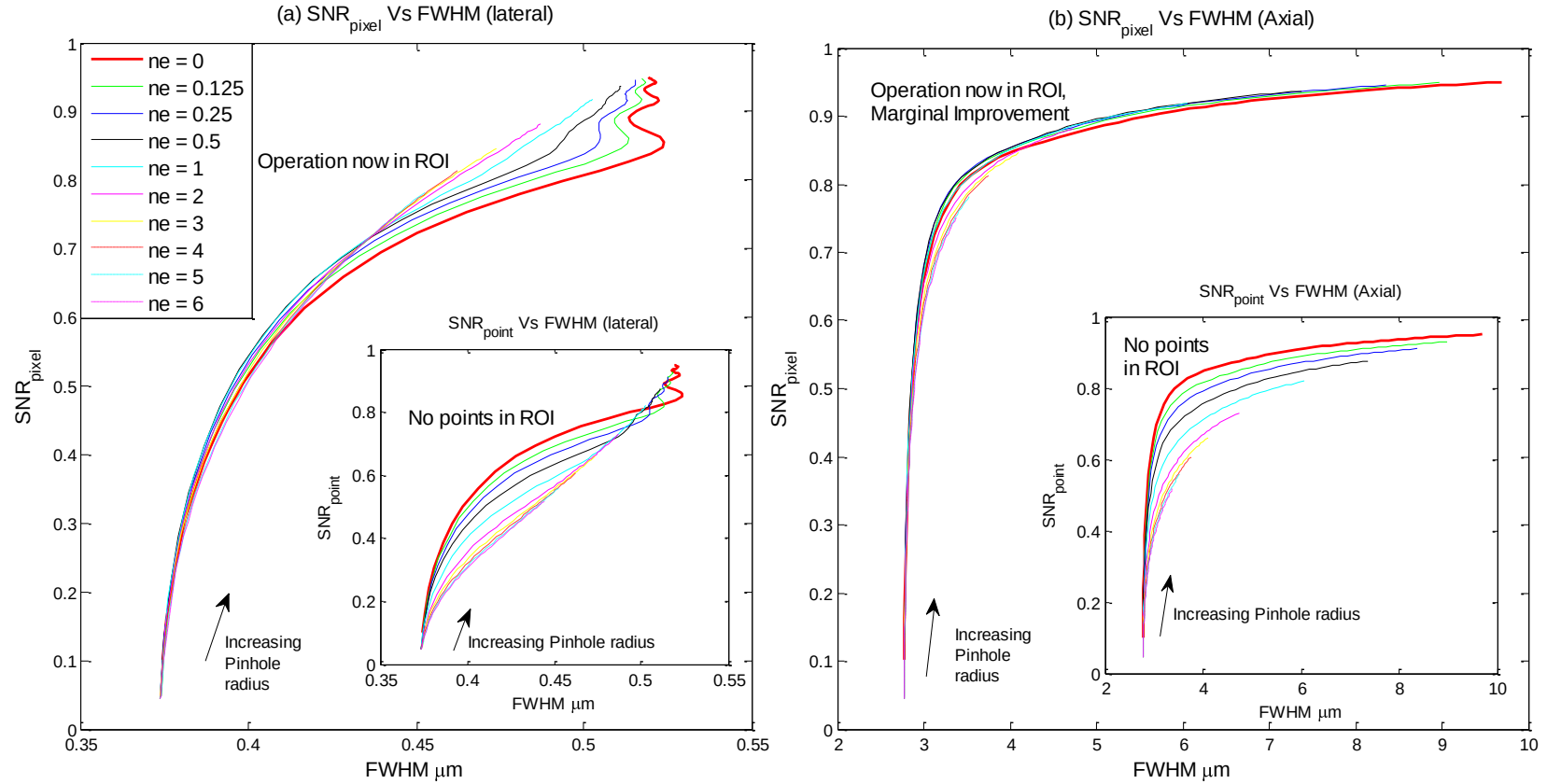


Figure 4.4: SNR-resolution trade-off along the lateral and axial directions respectively for pixel array detector based CFM system employing a ‘family’ of pinholes defined by equation 4.10. A better CFM SNR-resolution trade-off is obtained simultaneously in both lateral and axial directions. Insets show SNR-resolution trade-off using the same pinhole geometry in conventional (variable pinhole) point detector based CFM systems. Arrows indicate direction of increasing pinhole radius.

Examining plots in figure 4.4(a) and (b), it can be seen that higher values of ne result in a better SNR-resolution trade-off for larger pinhole radii, while smaller values of ne offer a better trade-off at smaller pinhole radii. In the case of a conventional (variable pinhole) point detector based CFM system, irrespective of the pinhole radius, the performance degrades with increasing ne (see inset of figures 4.4(a) and (b)). This suggests that the circular pinhole geometry may be the optimum choice for a conventional point-detector based CFM system.

At smaller pinhole radii and when $ne > 2$ the pixel array detector based CFM system underperforms the conventional CFM systems. This can be inferred from figure 4.4; the curves in yellow ($ne = 3$) fall under the bold red curve (conventional circular pinhole base CFM). Thus in order to realise simultaneous performance gains in both the lateral and axial directions, ne between 0 and 2 should be chosen. Pinhole geometry with $ne = 1$ thus offers the best SNR-resolution gains over the entire range of pinhole radii. For simplicity, here on in this thesis this pinhole will be referred to as a triangular pinhole.

To illustrate the effect of the performance gains on imaging results, figure 4.5 demonstrates the lateral resolution improvement achieved using a pixel array detector based CFM system employing the triangular pinhole geometry at various SNR values. Here the simulated object is an infocus 2D object consisting of two concentric rings of width $1\text{ }\mu\text{m}$ separated by $4\text{ }\mu\text{m}$. Although 3D resolution improvement is achieved across all SNR values, in the simulated images shown at $\text{SNR} > 0.81$ the inner ring of the sample planar object begins to blur into a single circular spot in the conventional system, but

is still distinguishable (as a ring structure) under the new detection scheme. In comparison to the conventional CFM system, at an SNR of 0.81, the new detection scheme offers a lateral resolution improvement of about 10%.

Figures 4.6 and 4.7 illustrate the axial resolution improvement offered by the new detection scheme at two pinhole radii - 0.25 μm and 0.6 μm . Here the simulated object is the same 3D structure shown in figure 3.9. The rejection of light in the out-of-focus planes at both the pinhole radii is demonstrated here. There is a noticeable decrease in the intensity of the lower images which is due to the smaller pinhole area of the triangular pinhole in comparison to the conventional circular pinhole. At 0.25 μm the new detection scheme offers an axial resolution improvement of just 1.5% with an SNR degradation of 1%. At a larger and more conventionally used pinhole radius of 0.6 μm , an axial resolution improvement of 14% is obtained with an SNR degradation of 9% (i.e. axial resolution gain about 1.5 times the SNR loss).

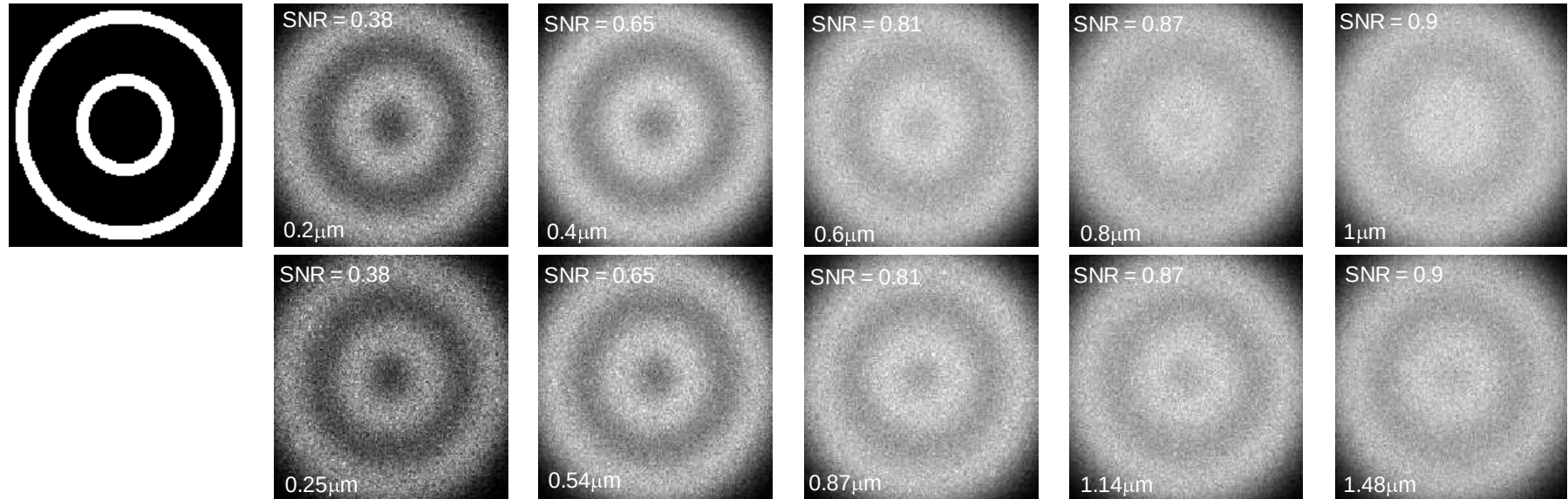


Figure 4.5: Simulated CFM images of a sample planar object (leftmost image, top row), at various SNR values, using a triangular-pinhole-pixel-array detectors based systems (bottom row) and a conventional circular-pinhole-point-detector based system (top row). The planar object consists of two concentric rings each of $1\mu\text{m}$ width and separated by $4\mu\text{m}$. Here $\text{NA} = 0.5$ and $\lambda = 500\text{ nm}$. Each image has been normalised by its maximum intensity.

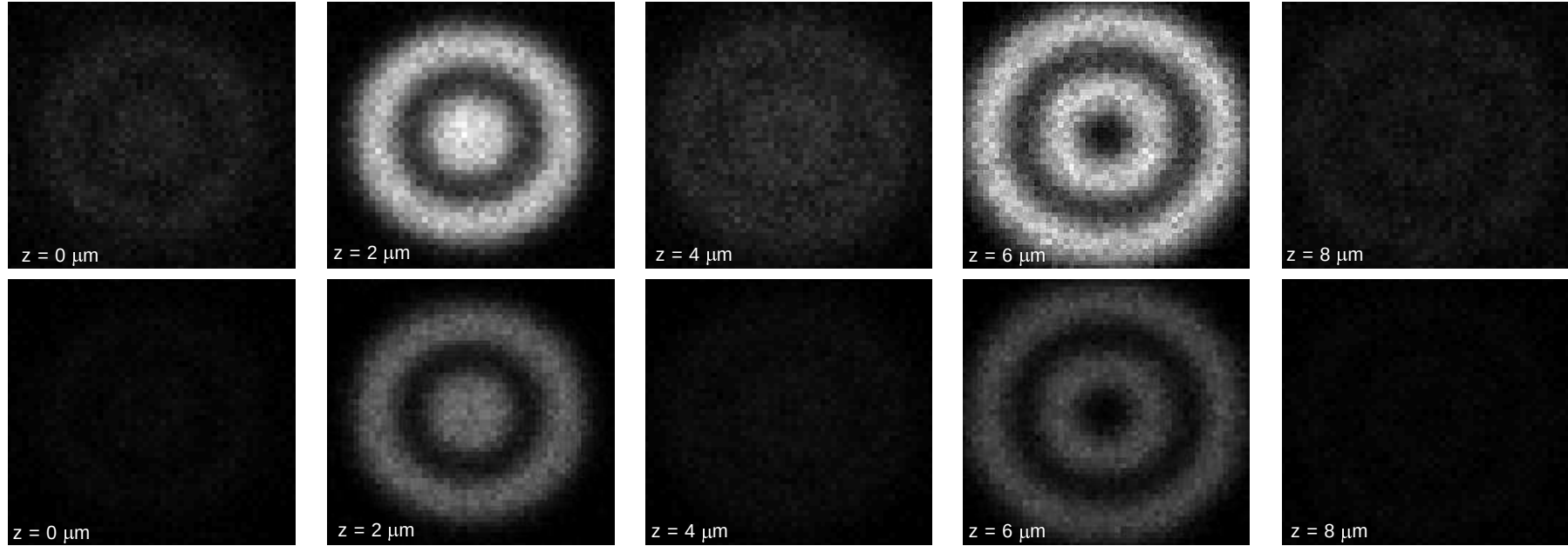


Figure 4.6: Simulated CFM images of a sample object (see figure 3.9, top row) as it is moved relative to the focal plane, at a pinhole radius of $0.25 \mu\text{m}$ using a conventional circular-pinhole-point-detector system (top row) and a $0.25 \mu\text{m}$ triangular-pinhole-pixel-array detector (bottom row). Here $\text{NA} = 0.5$ and $\lambda = 500\text{nm}$. Each image has been normalised by the maximum intensity of all 10 images.

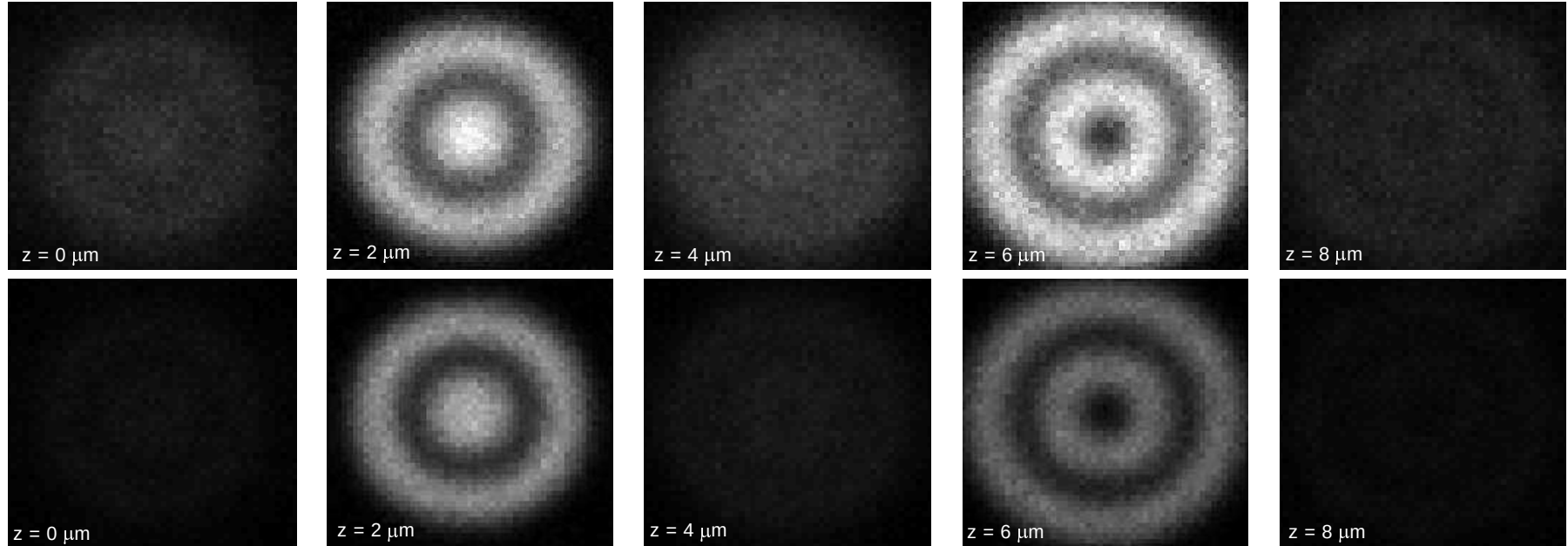


Figure 4.7: Simulated CFM images of a sample object (see figure 3.9, top row) as it is moved relative to the focal plane, at a pinhole radius of $0.6 \mu\text{m}$ using a conventional circular-pinhole-point-detector system (top row) and a $0.6 \mu\text{m}$ triangular-pinhole-pixel-array detector (bottom row). Here $\text{NA} = 0.5$ and $\lambda = 500 \text{ nm}$. Each image has been normalised by the maximum intensity of all 10 images.

4.2.2 Generalised normal (GN) distribution based pinhole family:

Theoretically, an unlimited number of pinhole geometries or pinhole ‘families’ may be defined, in a fashion similar to equation 4.10, that approximate to a circular pinhole at a given shape parameter. While the triangular pinhole geometry demonstrated performance gains along both directions, the gain was more substantial in the lateral direction. The next geometry will demonstrate how at the cost of reduced lateral performance, better gains can be made in the axial direction. Consider a GN distribution based pinhole family, defined mathematically in equation 4.11, which approximates to a circular pinhole as $ne \rightarrow \infty$.

$$Pin(v, v_d) = \begin{cases} \left(\frac{e^{-\left(\frac{v}{v_d}\right)^{ne}} - e^{-1}}{1 - e^{-1}} \right) & \text{for } v \leq v_d \\ 0 & \text{otherwise} \end{cases} \quad (4.11)$$

This pinhole geometry for $\frac{1}{4} \leq ne \leq 32$ has been plotted in figure 4.8 both in 1D and 2D.

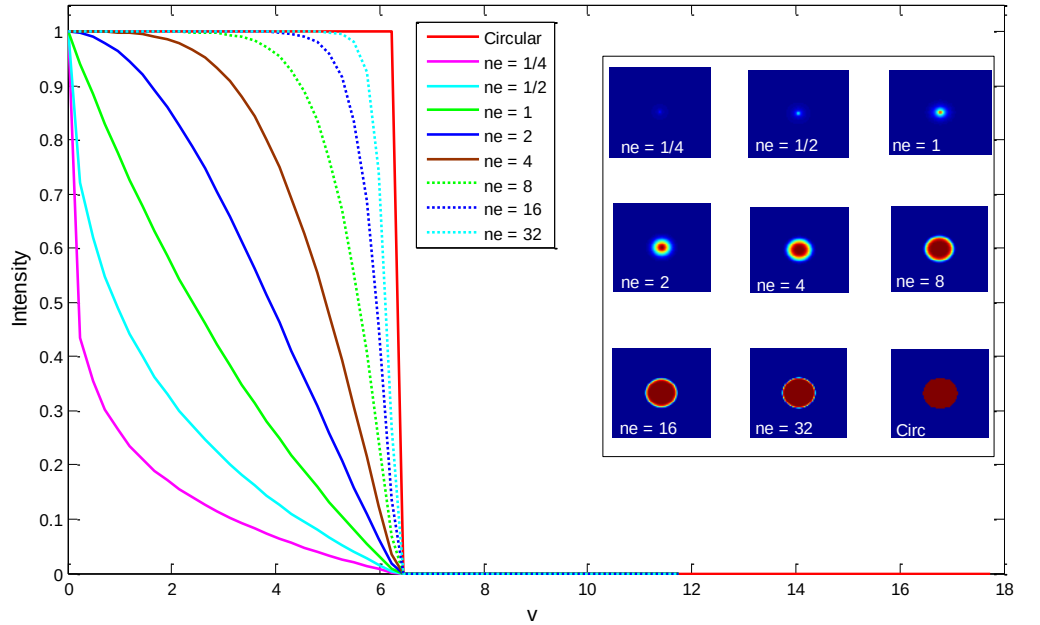


Figure 4.8: GN distribution based pinhole geometry shown at several values of shaping parameter ne . Inset shows 2D visualisation of the same along with the circular pinhole visualisation for reference. Here $v_d = 2\pi$

For the various values of ne , resolution (lateral and axial FWHM) and SNR as a function of pinhole radius for a pixel-array-based detector as well a conventional point-detector-based CFM system are shown in figure 4.9. As expected, for very large ne , the resolution (lateral as well as axial) and SNR characteristics of the CFM system mimic that of a conventional CFM system. Increasing ne improves the 3D resolution but degrades the SNR. Interestingly as ne is increased, at small pinhole radii ($< 0.61 \mu\text{m}$) the pixel array detector based CFM system offers better lateral than axial resolution improvement. Examining the trade-off curves in figure 4.10 and 4.11, it can be seen that choosing $ne = 4$ offers maximum and simultaneous performance gains in the lateral and axial directions. Thus shape parameter $ne = 4$ can be considered to be a good choice for the GN distribution based pinhole family.

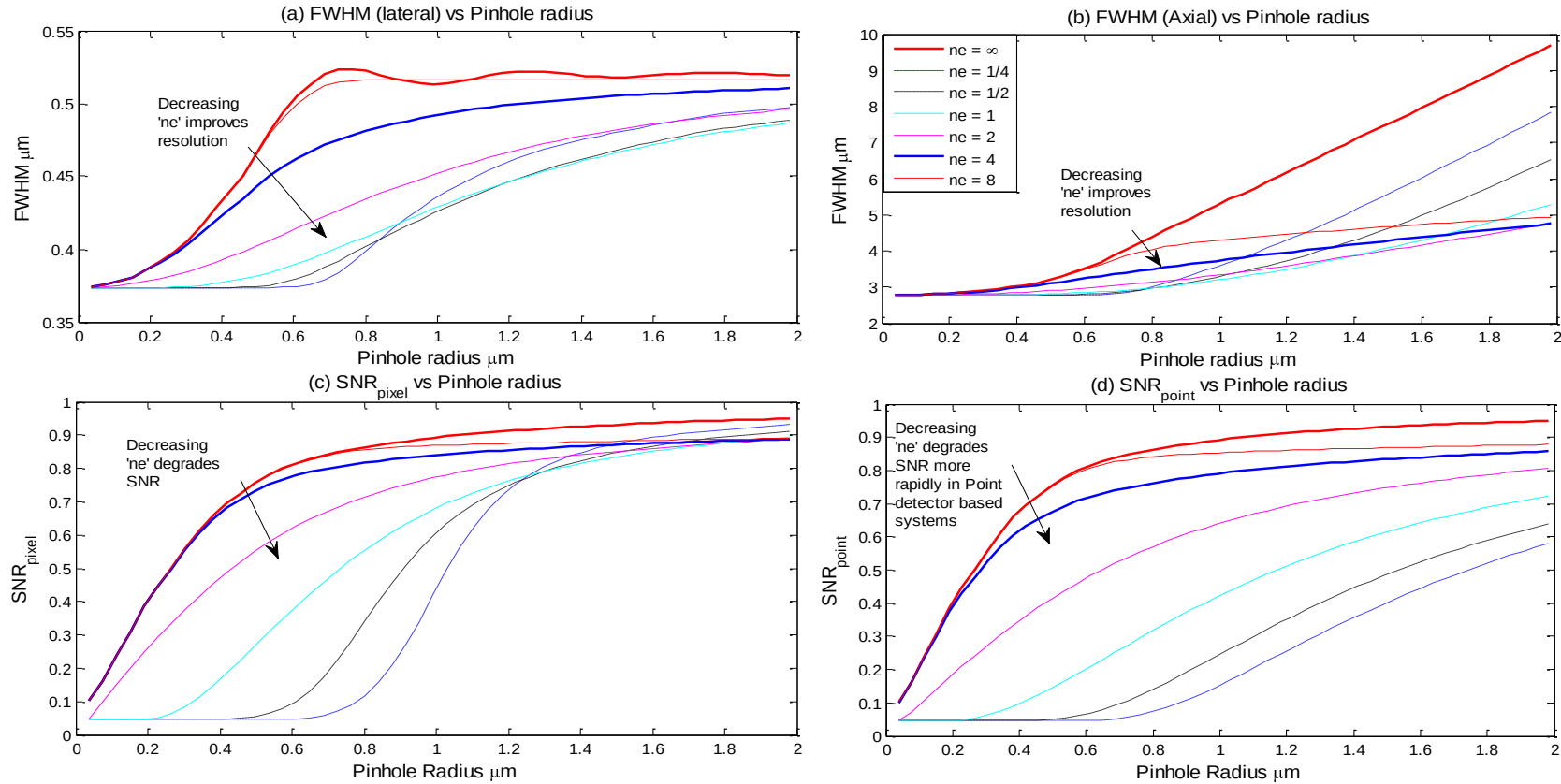


Figure 4.9: Effect of parameter ne (for the GN based pinhole defined in equation 4.11) on FWHM of confocal PSF in the (a) lateral and (b) axial directions. Effect of parameter ne on the SNR of a (c) pixel array based detector and (d) point detector based CFM system. Here $NA = 0.5$ and $\lambda = 0.5 \mu\text{m}$.

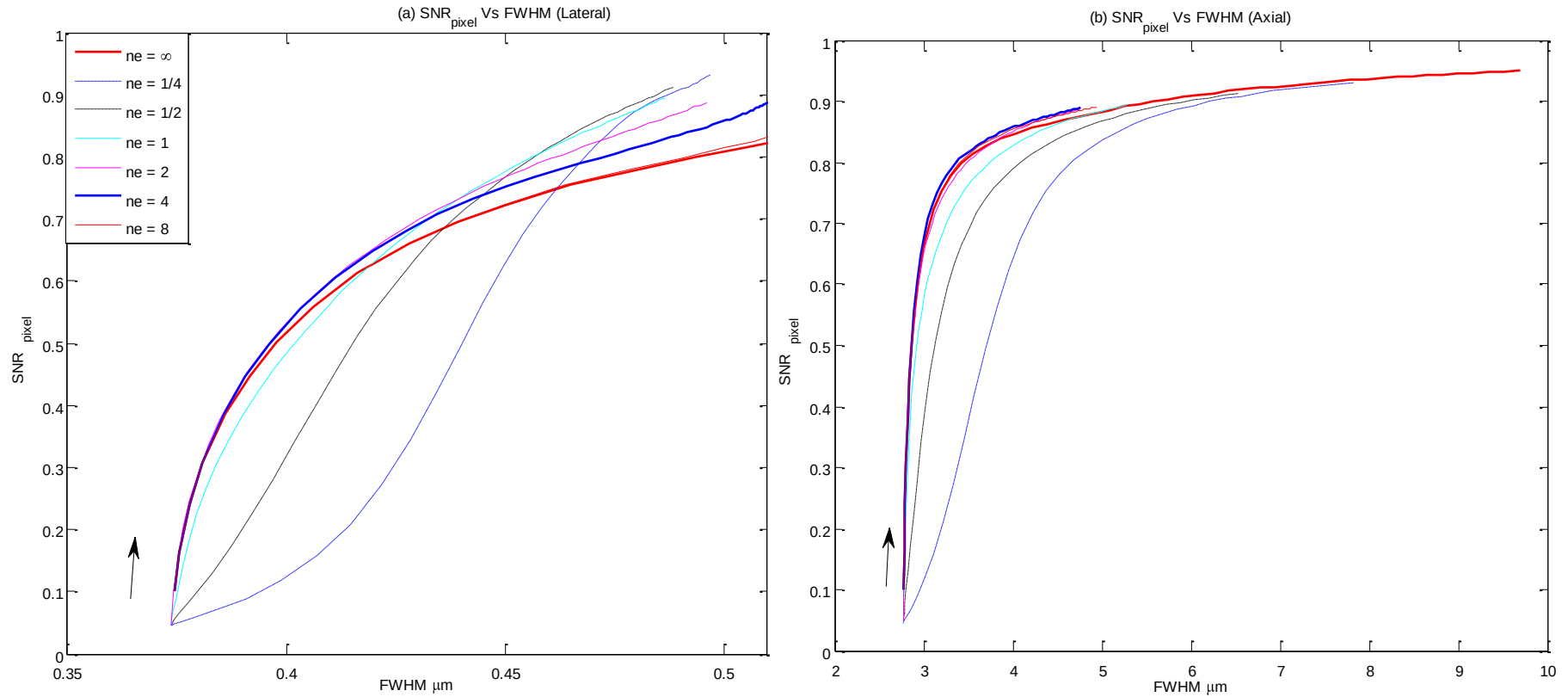


Figure 4.10: SNR-resolution trade-off along the lateral and axial directions respectively for pixel array detector based CFM system employing a ‘family’ of pinholes defined by equation 4.11. Arrows indicate direction of increasing pinhole radius.

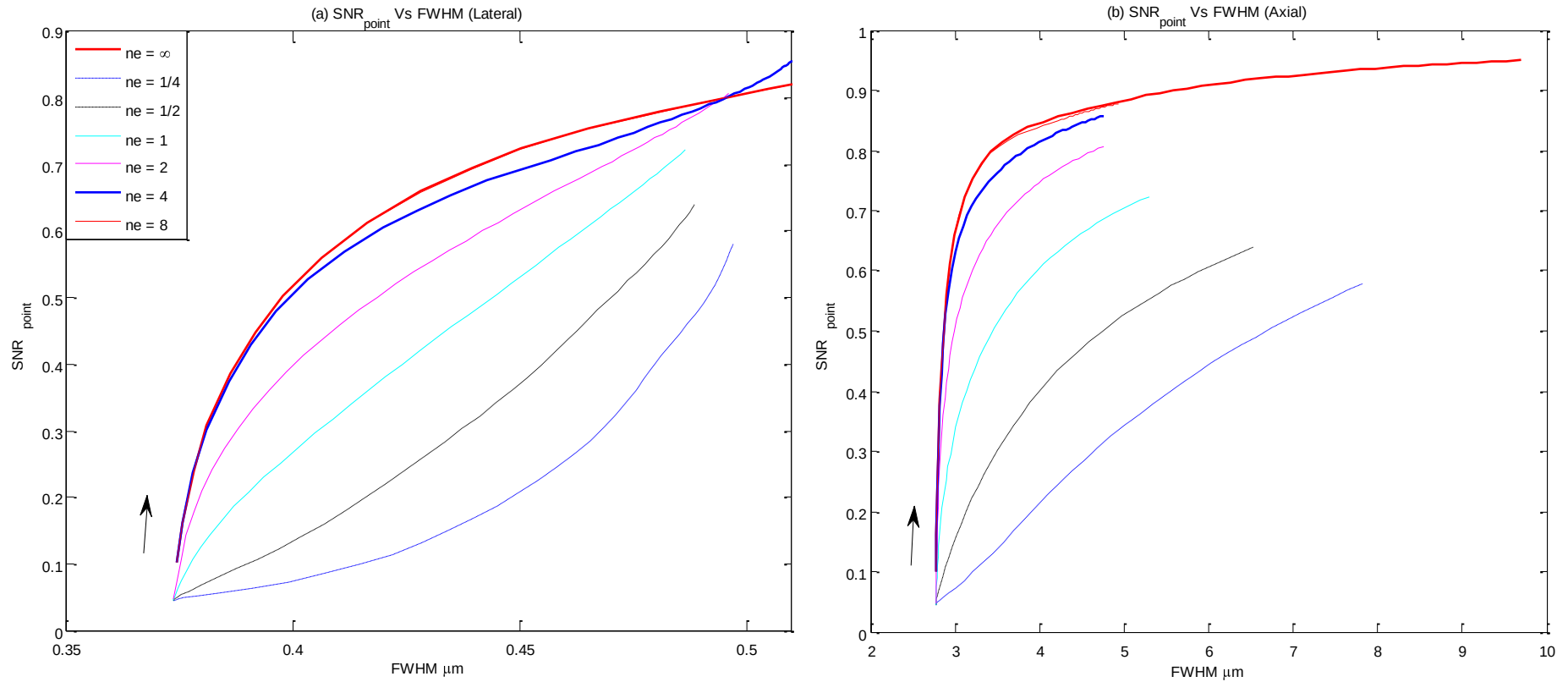


Figure 4.11: SNR-resolution trade-off using the same pinhole geometry used in conventional point detector based CFM systems. Arrows indicate direction of increasing pinhole radius. Arrows indicate direction of increasing pinhole radius.

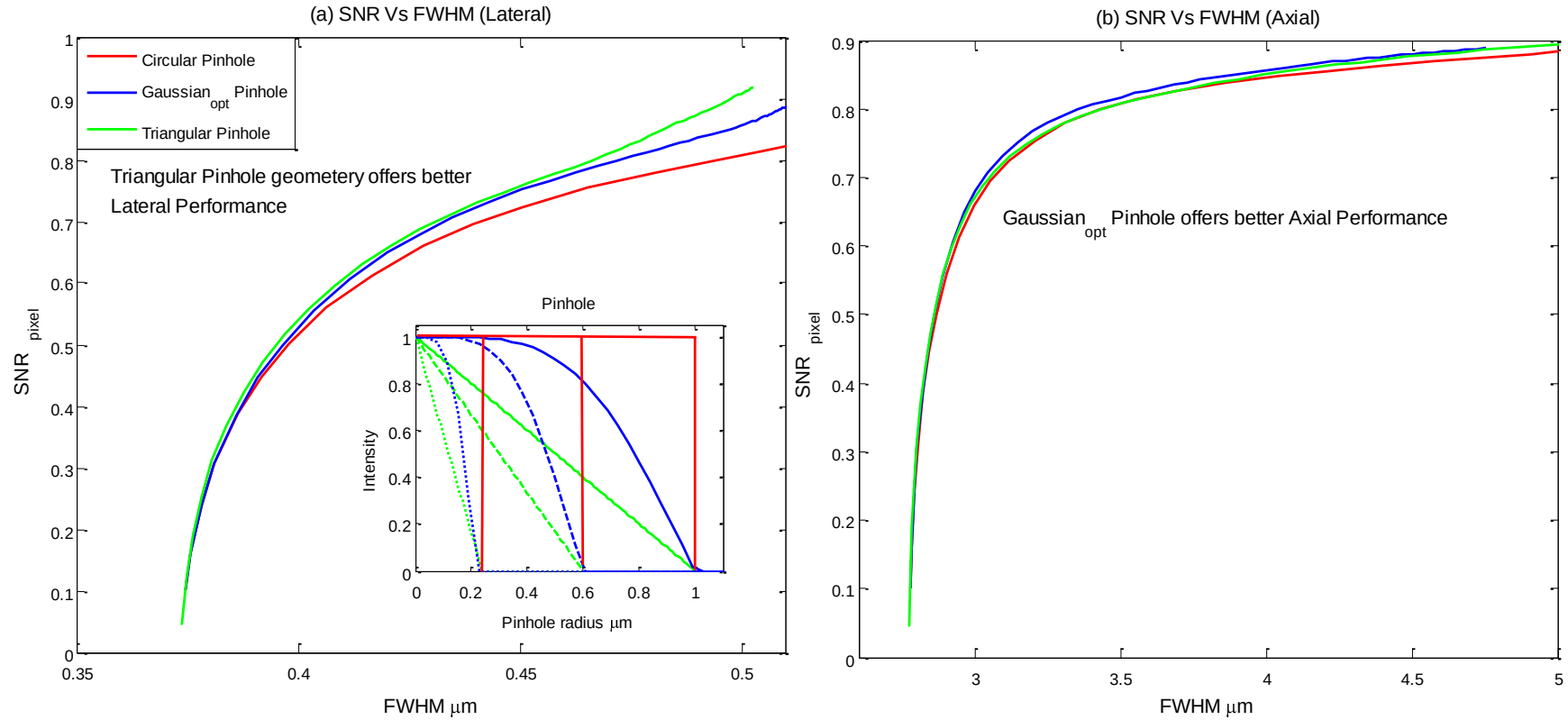


Figure 4.12: Comparing two pinhole geometries – Triangular and GN based ($ne = 4$) that provide better SNR-resolution trade-offs (over conventional circular pinhole-point detector based CFM system) along either directions when used in a pixel array detector based CFM system. Inset in (b) shows the 1D pinhole geometries for the two pinholes for three values of pinhole radius. Curves in blue represent the GN pinhole geometry with $ne = 4$, curves in green represent the triangular pinhole ($ne = 1$) and curves in red represent the conventional circular pinhole.

The triangular and GN distribution pinholes ($ne = 4$) when used in conjunction with pixel array detectors have thus been shown to offer simultaneous resolution gains along both the lateral and axial directions – but it is of interest to see which is the better of the two. To answer this question, the trade-off curves for the two pinhole types shown in figure 4.12 are examined. At larger pinhole radii ($> 0.61 \mu\text{m}$) it can be observed that the GN pinhole ($ne = 4$) based CFM system offers better performance gains (over conventional point detector CFM systems) in the lateral direction, while the triangular pinhole based system offers better performance improvements in the axial direction. However at smaller pinhole radii, the triangular pinhole based CFM system outperforms the GN pinhole ($ne = 4$) system in both lateral and axial directions.

4.3 Genetic algorithm based optimisation of pinhole geometry

The last section demonstrated that alternative pinhole geometries can offer improved SNR-resolution trade-off when employed in conjunction with pixel array detectors. The results indicate that several non-circular pinhole shapes possibly exist that could offer performance gains in CFM. A rigorous approach to finding an optimum pinhole shape or a set of optimum pinhole shapes would require an intensive computer aided search of all possible pinhole profiles.

4.3.1 Multi-objective optimisation problem

From results presented earlier in this chapter (and in chapter 3) it should be apparent that SNR is inversely proportional to both lateral and axial resolution as the pinhole radius is increased. The ‘objectives’ – resolution and SNR are

thus conflicting, preventing simultaneous optimisation of each objective. This thus presents a multi-objective (MO) optimisation problem. Genetic algorithms (GAs) are a popular meta-heuristic that are particularly well suited for these class of problems. They are often customised to solve MO optimisation problems by using specialised fitness functions and introducing techniques for solution diversity. Traditionally, MO optimisation can be undertaken by either combining all the objectives into one single objective function or moving all but one objective to the constrained set [1]. The former approach is often undertaken by a weighted-sum method also known as scalarization technique [2] . The problem here lies in deciding appropriate weights (and scaling them) to factor the decision maker's preferences [3]. This can be difficult even for someone familiar with the problem domain. The latter approach, of converting all but one objective into the set of constraints, has the problem of deciding the appropriate value for the constraints (rather arbitrary) for the former objectives. Both these MO optimisation methods return single solution rather than a set of solutions.

In the current optimisation problem it is more interesting to obtain a set of solutions (i.e. set of pinhole shapes), that serve as solutions for the SNR-resolution trade-off, than a single solution. An approach to achieve this is to compute a Pareto optimal solution set. A Pareto optimal set represents a set of non-dominated solutions. Non-dominated means there is always some loss in one or more objectives to achieve gains in other objectives. The size of the Pareto optimal set is directly proportional to the number of objectives and the shape of the Pareto front (viz., the image of all efficient solutions [4]) indicates the nature of the trade-off between the various objectives. An

example of a Pareto curve is shown in figure 4.13. Here the points between points p_2 and p_5 define the Pareto front and are called non-inferior or non-dominated points. Points p_1 and p_6 are considered weak Pareto optimal points – non-inferior points but those that gain only across a particular objective. In the two-objective problem shown in figure 4.13, moving p_1 to p_2 for example offers gain only along $f_1(x)$ but no gain (or loss) along $f_2(x)$. Points p_2 , p_3 , p_4 and p_5 are considered strong or strict Pareto optima as moving between them results in gain in one objective and loss in the other.

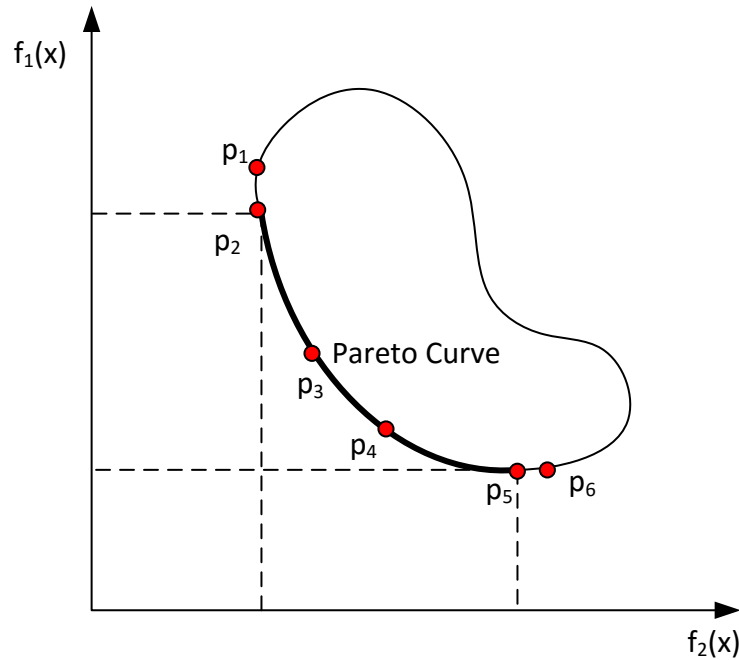


Figure 4.13: An example of Pareto curve for a two-objective $[f_2(x), f_1(x)]$ minimisation problem. Points p_1 and p_6 are weak Pareto optima; points p_2 , p_3 , p_4 and p_5 are strict Pareto optima.

Several MO optimisation GAs that compute Pareto-optimal solutions have been developed over the past two decades [5-16]. One of the first GAs developed is the non-dominated sorting genetic algorithm (NSGA) [10]. Kalanmoy Deb et al [16] in 2002 addressed some of the issues in NSGA such as the lack of elitism (loss of good solutions once they are found), need for sharing parameter and high computational complexity (proportional to the

cube of the population size) to propose the so called NSGA-II [1]. This fast and elitist MO evolutionary algorithm has been since incorporated as a function called gamultiobj in a recent release of MATLAB (see [17] for function format). Using the gamultiobj, the following section will present a set of Pareto optimal pinhole designs for the pixel-array detector based CFM system.

4.3.2 Defining a generic pinhole

Consider a pinhole visualised (in 1D) as shown in figure 4.14, with a large pinhole radius of $1\text{ }\mu\text{m}$. For the CFM system assume $\text{NA} = 0.5$ and $\lambda = 0.5\text{ }\mu\text{m}$.

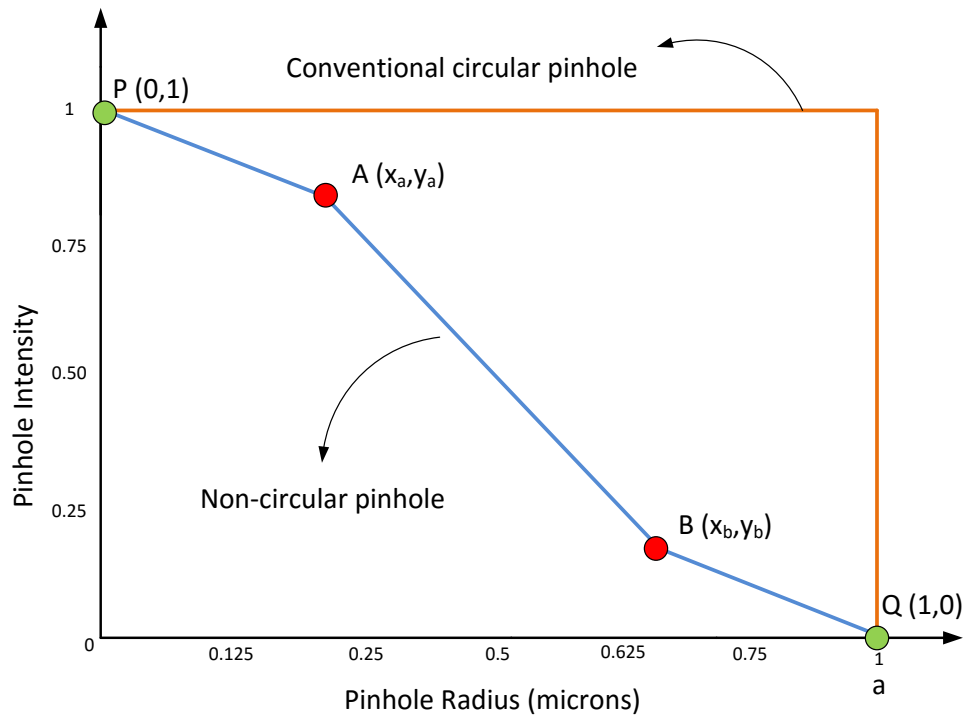


Figure 4.14: 1D pinhole function consisting of three lines PA, AB and BQ. P and Q are fixed points. $A(x_a, y_a)$ and $B(x_b, y_b)$ represent any point contained within the circular pinhole area and such that $x_b > x_a$ (necessary condition for a valid pinhole shape).

It may be noted that for a smooth pinhole function, a very large (ideally infinite) number of points need to be considered between P and Q but here the analysis is restricted to only two for computational reasons. For the two point

three line-segment pinhole shown in figure 4.14, the pinhole shape can be completely defined by four variables- x_a , y_a , x_b and y_b .

4.3.3 Defining the fitness function

Associated with a given variable set (i.e. a pinhole shape) are unique values of resolution (FWHM measure along the lateral and axial directions) and SNR. The performance a CFM system can thus be represented by the vector,

$$P_{CFM} = [FWHM_{lat} \ FWHM_{ax} \ SNR], \quad (4.12)$$

such that it is desirable to minimise,

$$P_{CFM} = [FWHM_{lat} \ FWHM_{ax} \ -SNR]. \quad (4.13)$$

P_{CFM} represents the MO GA fitness function. The two resolutions objectives $FWHM_{lat}$ and $FWHM_{ax}$, can be condensed into a single objective through a simple weighted sum approach,

$$FWHM_{3D} = w_{lat}FWHM_{lat} + w_{ax}FWHM_{ax}. \quad (4.14)$$

And thus the minimisation problem can be reduced to a two-objective optimisation problem,

$$P_{CFM} = [FWHM_{3D} \ -SNR]. \quad (4.15)$$

As mentioned previously, the weighted-sum approach has the disadvantage that the choice of weighting parameters can be arbitrary but offers substantial computation time advantage. Choosing equal weights i.e. $w_{lat} = w_{ax} = 0.5$, under the constraint $w_{lat} + w_{ax} = 1$ and $w_{lat} \geq 0, w_{ax} \geq 0$, here indicates an equal preference for lateral and axial resolution which can be considered reasonable in microscopy. However based on the target application, relative values of these parameters can be set differently. For example in particle

tracking or axial displacement measurement axial response sensitivity is more important than lateral resolution [18].

4.3.4 Pareto optimal pinhole design

The vector defined in equation 4.15 represents a two-objective four variable fitness function. Using MATLAB's inbuilt multi-objective optimisation function gamultiobj it is possible to produce non-dominated Pareto optimal pinhole shapes for the pixel array detector based CFM system. With an initial population of 50 and 200 generations, 20 non-dominated Pareto optimal points are calculated as seen in figure 4.15.

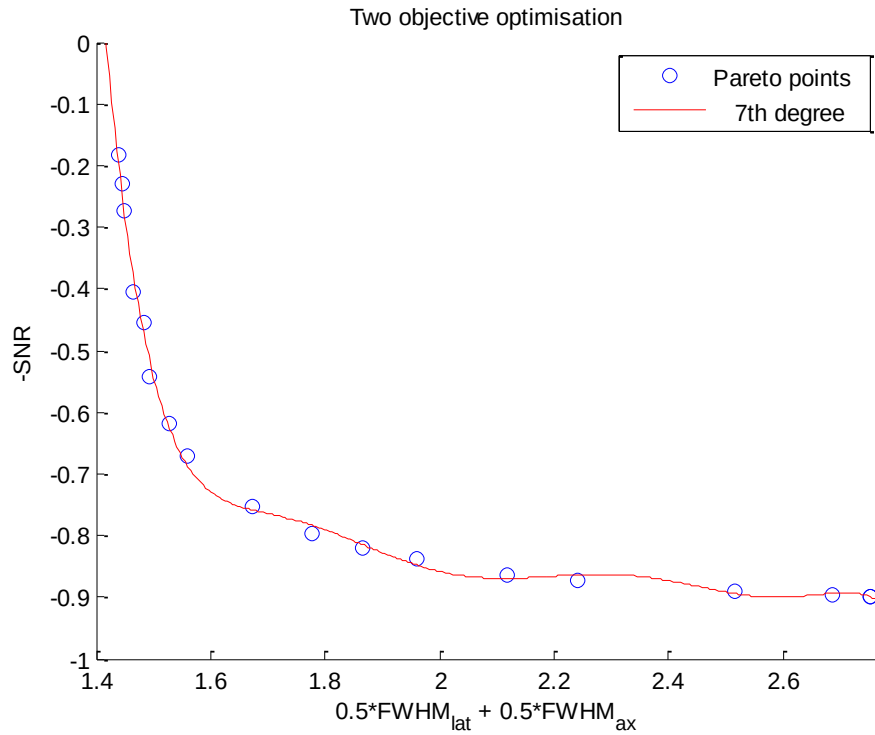


Figure 4.15: Pareto front (7th degree polynomial fit to the Pareto optimal points is shown in red) from the resolution-SNR performance optimisation of a pixel-array based CFM system using a generic pinhole shown in figure 4.14.

Each of the points corresponds to a unique pinhole shape. Some of these shapes have been plotted in figure 4.16. It can be observed that each shape is characterised by a large amplitude (~ 1) within a small pinhole radii and a

decreasing amplitude at larger pinhole radii. The slope of the edge of the pinhole shape depends on the pinhole radii – a sharp edge for smaller pinhole radii and a more gradual decline for larger pinhole radii.

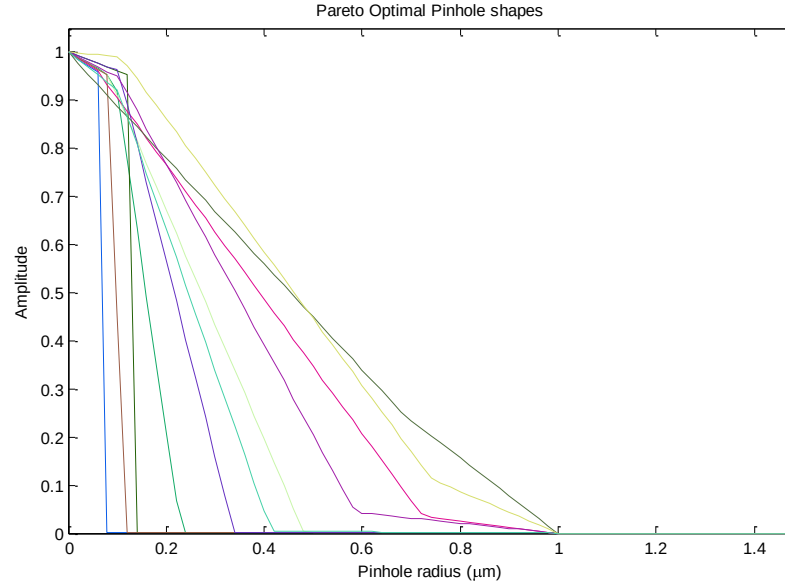


Figure 4.16: Pareto optimal pinhole shapes for a pixel array detector based CFM system using a generic pinhole as shown in figure 4.14.

By comparing the Pareto optimal pinhole shapes with the triangular and optimum GN pinhole geometry (examined earlier), the similarity in the shapes should be apparent. Thus by choosing equal weights for lateral and axial resolution, an improved SNR-resolution trade-off in either direction can be demonstrated. It is also possible to generate different optimal pinhole shapes with different weighting parameters based on the user's preference of one resolution metric over the other.

Based on the results presented in this chapter, the performance gain achieved using non-circular pinhole geometries in a pixel array-detector based CFM system is not very substantial. The next chapter will investigate the merit in returning to the point detector CFM system by using alternative detection arrangements.

4.4 Chapter Summary

- One of the key components is the detection pinhole; it is its geometry that is responsible for setting the operating point on the SNR-resolution trade-off curve.
- An alternative to using single point detectors in CFM systems is to use pixel array based detectors which offer significantly improved SNR when used in conjunction with new pinhole geometries.
- Two pinhole geometry families can serve better SNR-resolution trade-off over conventional circular pinhole-point detector based systems, as demonstrated by the use of a triangular pinhole and the GN based pinhole.
- For a pixel array detector based CFM system, a triangular pinhole profile was shown to offer simultaneous performance gains along both the lateral and axial directions.
- However, the improvement is marginal. In order to realise substantial improvements, alternate detection schemes and image reconstruction methods must be employed. The former approach shall be implemented in the next chapter although with a point detector.

4.5 References

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CHAPTER 5

CFM performance enhancement using a differential pinhole

As discussed in chapter 3, pinhole size and geometry play an important role in deciding the performance of a CFM system. Applications demanding a high SNR require a large pinhole and this invariably impedes resolution. The pinhole geometry directly impacts the shape of the 3D PSF; experimenting with other pinhole profiles can potentially offer a more substantial SNR-resolution trade-off than demonstrated using the earlier geometries. In this chapter we investigate the use of an alternative pinhole geometry – a differential pinhole in CFM. This involves separately detecting two signals, one from a central circular pinhole and the other from an annular region around the central region; this we designate as a ‘differential’ pinhole. The idea of using a differential pinhole has been discussed by various authors [1-4]. In references [1-3], physical pinholes are suggested, whereas in reference [4] the same concept is suggested but using a CCD detector array to emulate the pinholes. Segawa, Kozawa and Sato [5] discuss subtractive techniques for more general dual PSF microscopes, but the implementation of these is more complex requiring special polarised illumination.

The aim of this chapter is to investigate the effect of varying the parameters describing a differential pinhole on resolution and SNR. Optimum parameters are identified and it is shown that the signal resulting from this design provides a significantly improved SNR-resolution trade-off than that offered by either a conventional circular pinhole based

CFM system or the designs considered in chapter 4. The impact of background noise will also be investigated. The chapter will also demonstrate the advantage of using a differential pinhole in improving the performance of a conventional circular-pinhole based CFM system that employs an alternative pupil filter.

5.1 Defining a Differential Pinhole

Consider an elliptical shaped pinhole (figure 5.1(b)) tilted an angle of 45 degrees as seen in figure 5.1(a) whose projection in the plane perpendicular to the optical axis (see figure 5.1(c)) assumes a circular shape defined mathematically as,

$$Pin(r) = \begin{cases} 1 & \text{for } r < r_i \\ -\gamma & \text{for } r_i < r < r_p, \\ 0 & \text{elsewhere} \end{cases} \quad (5.1)$$

where r_p is the pinhole radius, r_i is the radius of the inner transparent annuli and γ is the multiplication factor applied to the photon counts from the second detector. Note that a similar output would be achieved by using a reduced reflectivity for the outer annulus but this would give an inferior SNR in the subtracted signal. This detector arrangement could be implemented practically as shown in figure 5.1(a) by allowing different parts of the light onto separate detectors. Notably this arrangement requires two detectors (i.e. increased system cost) and the 45 degree tilt of the design would cause the different sides of the pinhole to be in slightly different planes. An alternative approach to overcome the above problems would be to employ a detector array and to sum appropriate pixels in a similar way to the arrangement in [4]. This detector array approach has the advantage that many different pinhole sizes and regions used for image formation may be selected from a single measurement. The advantage of the detector approach shown in figure 5.1(a) is that fast

and high dynamic range detectors such as PMT's may be used. For confocal applications, such as fluorescence lifetime imaging microscopy (FLIM) or fluorescence (cross-) correlation spectroscopy (FCS/FCCS), that demand very high speed data acquisition, point detectors such as PMTs prove more suitable than CCDs [6]. Expanding on the ideas laid down by Bertero [7] and Sheppard[8], ZEISS recently introduced the LSM 880 microscope that employs the so called Airyscan detector [9]; 32 individual detector elements functioning as pinholes capture all available light from an Airy disk simultaneously. At the expense of a slightly increased implementation cost (from the use of a reflective pinhole and two point detectors), the detector arrangement in figure 5.1, can offer similar data acquisition speeds to a conventional CFM.

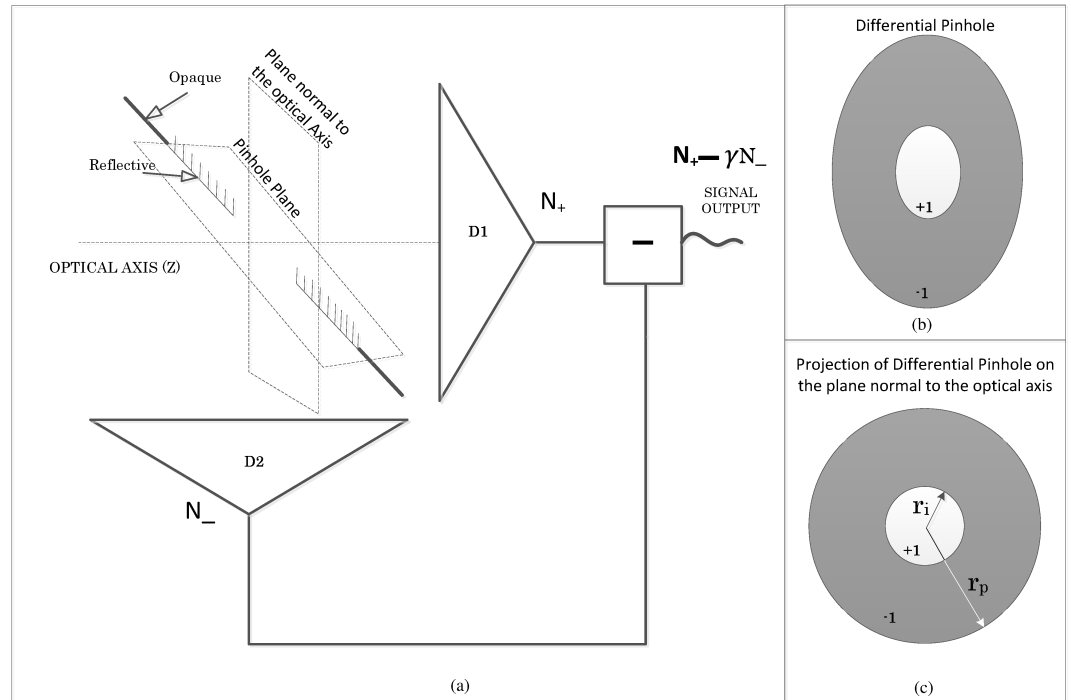


Figure 5.1: (a) Detector arrangement showing how a differential pinhole is realised using two detectors D1 and D2. (b) The Differential pinhole. (c) The pinhole's projection on a plane perpendicular to the optical axis is shown. D1 collects the photons incident within a pinhole radius r_i while D2 detects photons reflected within the region $r_i < r < r_p$. The scaled photon counts are subtracted to produce the final signal.

5.2 Calculating SNR

If N_+ and N_- denote the number of photons collected by detectors D1 and D2 respectively then the received signal S_{diff} is given by $N_+ - \gamma N_-$ and the total noise variance σ_{diff}^2 by the sum of the noise variances generated by D1 and D2. Thus,

$$\sigma_{diff}^2 = N_+ + \gamma^2 N_- \quad (5.2)$$

Assuming very weak sample auto-fluorescence and that poissonian shot noise is the chief noise contributor; the SNR of a CFM system using a differential pinhole is given by,

$$\text{SNR}_{diff} = \frac{S_{diff}}{\sqrt{\sigma_{diff}^2}} = \frac{N_+ - \gamma N_-}{\sqrt{N_+ + \gamma^2 N_-}} \quad (5.3)$$

The influence of sample autofluorescence will be investigated in section 5.6.

5.3 Calculating Resolution

Three parameters can be used to define the shape of the differential pinhole projection defined in equation (5.1): α , the ratio of the outer to the inner pinhole radius; γ , the multiplication factor applied to the photon count from the outer annulus; and r_i , the inner pinhole radius. This chapter is concerned with the optimum choice of these parameters to give a good resolution whilst maintaining a good SNR. It is useful to see how variations in these parameters affect the point-spread functions. Figures 5.2(a) and 5.2(c) show the effect of changing α and γ on the lateral confocal point-spread function. It may be seen that as α is increased the PSF mainlobe width reduces but at the expense of negative

sidelobes. A similar pattern of PSF changes is observed in figure 5.2(c) when the multiplication factor is increased.

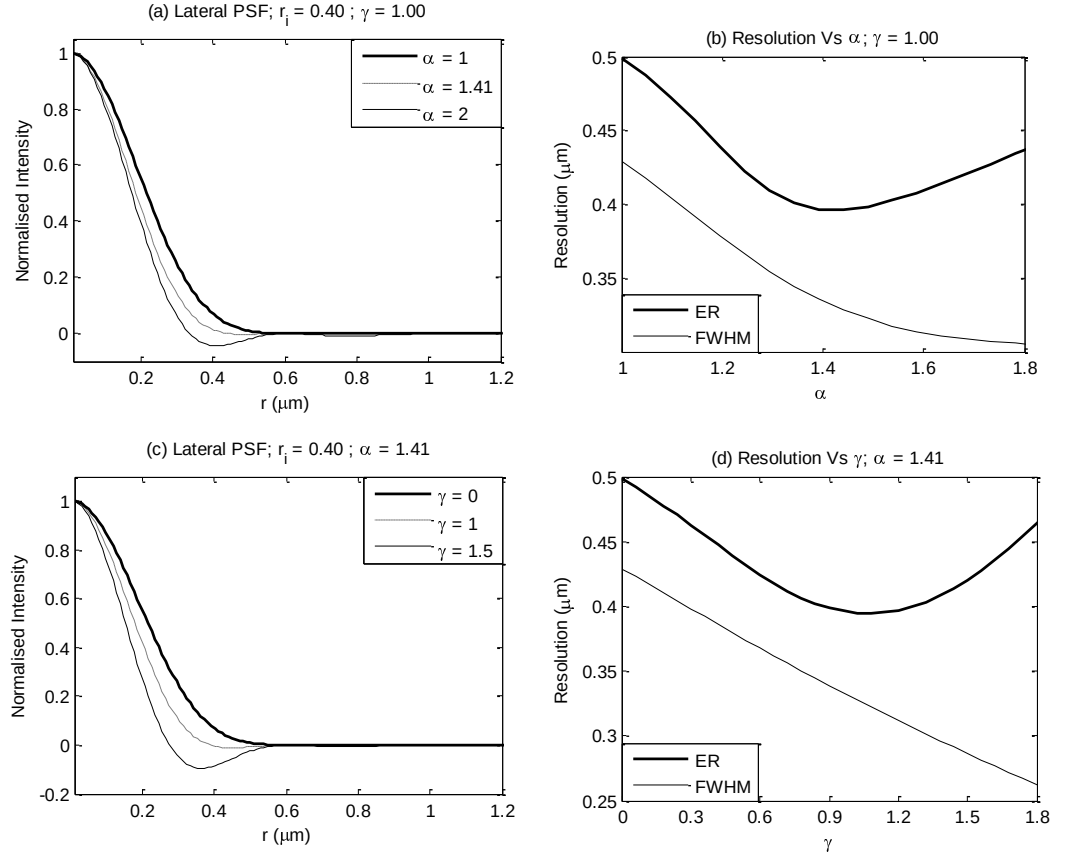


Figure 5.2: (a) and (c) show the effect of varying α and γ , respectively, on the lateral responses of a confocal fluorescence microscope employing a differential pinhole. Similar trends can be observed for the axial responses and thus are not shown here. (b) and (d) show the variation of lateral resolution as a function of α and γ , respectively, as defined by the ER metric and FWHM metric. Note how the negative sidelobes produced on increasing α or γ is accounted for only by the ER metric. Here $r_i = 0.4\mu\text{m}$, $\text{NA} = 0.5$ and $\lambda = 0.5\mu\text{m}$

Need for an alternative resolution metric: A commonly used measure of the resolution of an optical system is the width of the PSF as characterised by the full-width at half maximum (FWHM). The FWHM observed as α and γ are increased are plotted as the thinner lines in figures 5.2(b) and 5.2(d), respectively. It may be seen that, by this measure, the resolution appears to improve as α and γ are increased. However, clearly, this measure

does not take into account the presence of the increasing negative sidelobes visible in figures 5.2(a) and 5.2(c).

In order to take account of the negative effect of such sidelobes on imaging performance, an alternative measure of resolution designated as equivalent radius (ER) will be used. For lateral resolution the ER is defined as the radius of a circular top-hat function with a height of the maximum of the PSF and with the same volume as the modulus of the PSF. For the axial resolution ER is defined as the radius of a top-hat function with the height of the maximum of the axial response and with the same area as the modulus of the axial response. Mathematically, ER in the lateral direction ER_{lat} and axial direction ER_{ax} can be calculated as equations (5.3) and (5.4) respectively,

$$ER_{lat} = \sqrt{2 \int |PSF(v)| v dv} \quad (5.4)$$

$$ER_{ax} = \int |PSF(u)| du \quad (5.5)$$

and importantly, these measures take into account the presence of side lobes in the PSFs. These ER measures are plotted as the bolder lines in figures 5.2(b) and 5.2(d) and it may be seen that as α and γ increase the ER resolutions initially improve as the main lobe narrows but then deteriorate as the effect of the negative sidelobes increases.

5.4 Determining an optimum differential pinhole geometry

As the pinhole geometry is controlled by three parameters and the result of changing any of these parameters will, influence both the resolution and the SNR of the system; it is a complex task to try to find optimum conditions. To constrain the problem a constraint of always varying r_i to generate a chosen SNR value is imposed. This value has been set at 0.5; this means that the differential pinhole with selected α and γ values is assumed to be adjusted in overall radius so as to deliver a SNR of 0.5 (i.e. the same SNR as for a conventional confocal pinhole that admits 25% of the image-plane light). Constrained in this way, the resolution achievable for any α and γ values can be found. These are plotted (as contour diagrams) for the lateral and axial (ER) resolutions in figures 5.3(a) and 5.3(b).

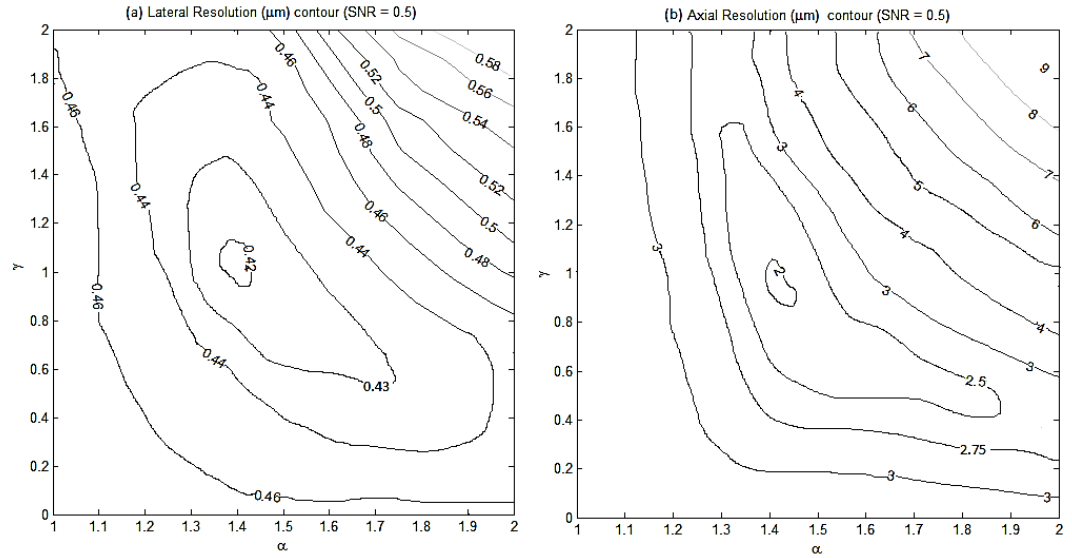


Figure 5.3: ER based resolution contours for a constant SNR of 0.5. Minima of the respective contours are found to be at $\alpha = 1.41$ and $\gamma = 1.00$. Here $NA = 0.5$ and $\lambda = 0.5 \mu m$

It may be seen, that in both the lateral and axial cases, a minimum is present in these plots, indicating that an optimum pairing of α and γ exists. It is convenient that the optimum positions in both plots are closely aligned at around the $\alpha = 1.41$ and $\gamma = 1$

positions. Similar plots for different SNR values (below about 0.63) also show minima in these positions as shown in figure 5.4.

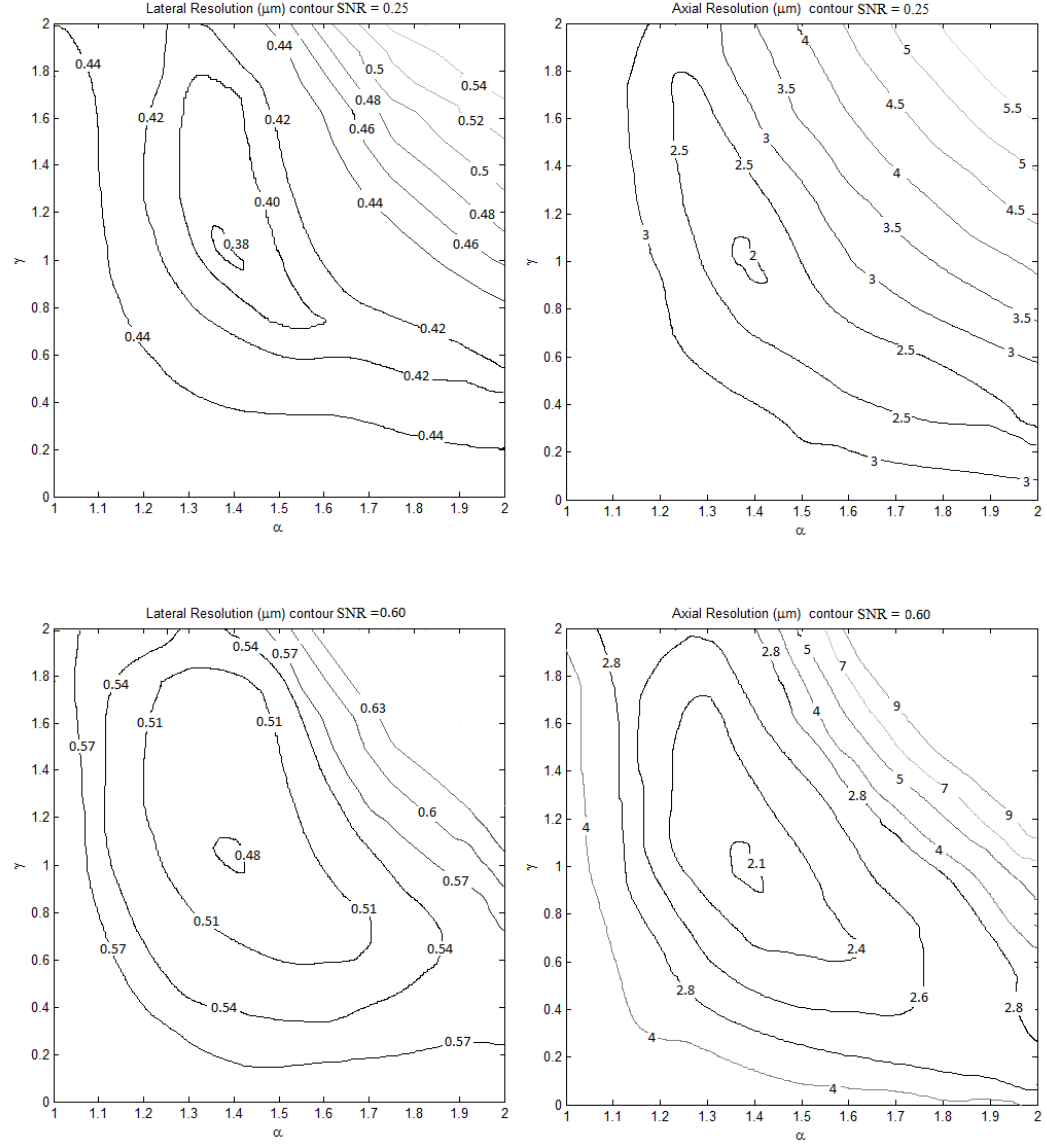


Figure 5.4: ER based resolution contours for a constant SNR of 0.25 (top row) and SNR of 0.6 (bottom row). Minima of the respective contours are found to be at $\alpha = 1.41$ and $\gamma = 1.00$. Here NA = 0.5 and $\lambda = 0.5 \mu\text{m}$

This suggests that these values i.e. $\alpha = 1.41$ and $\gamma = 1$ can be regarded as optimum in designing a differential pinhole. It is also interesting to examine the performance of a differential pinhole with these parameters by plotting the type of resolution vs

SNR characteristic plot shown in figure 3.13 and to compare with the plot for a conventional confocal pinhole. Figure 5.5 shows examples of these plots for the lateral and axial cases plotted both for the FWHM and the ER resolution criteria.

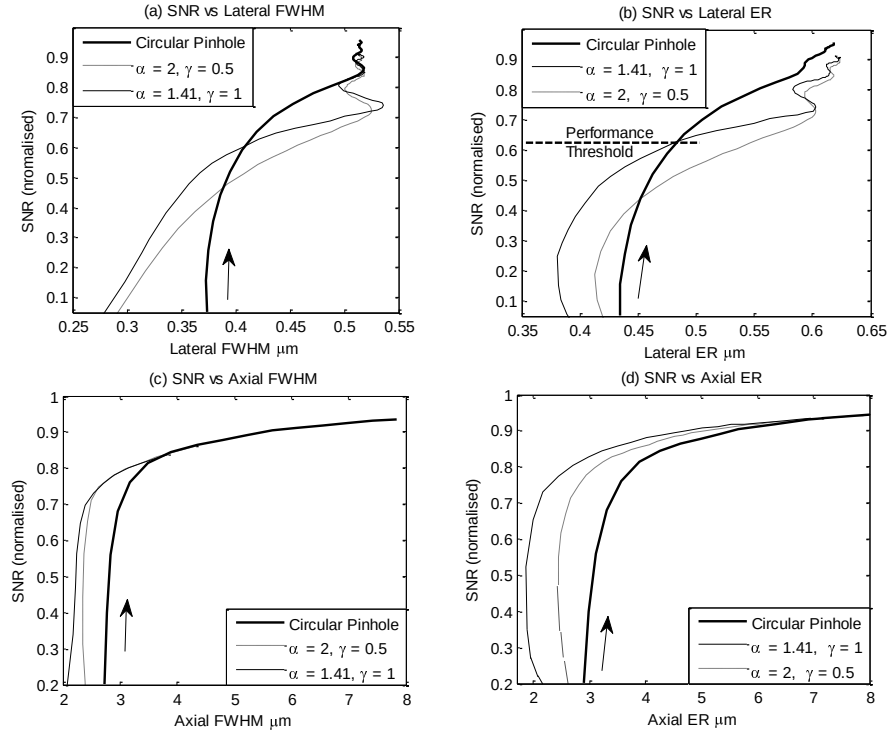


Figure 5.5: SNR Vs resolution characteristics based on FWHM (plots (a) and (c)) and ER (plots (b) and (d)). Arrows indicate direction of increasing r_i .

The bold dashed line in figure 5.5(b) marks the SNR threshold of 0.63 beyond which the differential pinhole based CFM system underperforms (worse SNR-resolution trade-off) with respect to a conventional (circular pinhole based) CFM system in the lateral direction. Below this threshold, the differential pinhole based CFM system offers maximum and simultaneous 3D resolution gains over conventional (circular pinhole based). This is realised with parameters $\alpha = 1.41$ and $\gamma = 1.00$. For any other parameter set chosen, the SNR of the differential pinhole system will always result in a lower SNR value than a conventional CFM system. However, it can be seen from figure 5.5(d), that the differential

pinhole system outperforms (better SNR-resolution trade-off) the conventional system, with the optimum parameter set, in the axial direction for all SNR values. It should be noted that the graphs presented in figure 5.5 are calculated for $NA = 0.5$ and for $\lambda = 0.5\mu\text{m}$. However the threshold value of 0.63 quoted for these parameters does not change significantly for different parameters. For example for $NA = 0.7$ and $\lambda = 0.4\mu\text{m}$, the threshold value changes to around 0.61.

5.5 Demonstrating the performance gain

To further illustrate the potential advantage of employing a differential pinhole system, computer simulated images have been generated to show the imaging performance predicted for some simple object forms. The images presented in this section have been generated using the commonly-used paraxial and scalar imaging approximations [10], which allow the use of discrete Fourier transforms to convert pixelated representations of pupil functions to point-spread functions; convolution operations were also implemented through Fourier transformation, multiplication and inverse transformation using the `fft2` function in MATLAB. The accuracy of the use of a Fourier optics approach has been discussed by a number of authors, see for example [11, 12]. Shot noise was modelled using the MATLAB Poisson noise generator `poissrnd`.

Figure 5.6 plots SNR versus outer pinhole radius for the two systems. It shows that based on the SNR desired, different pinhole radii would need to be chosen for the two systems.

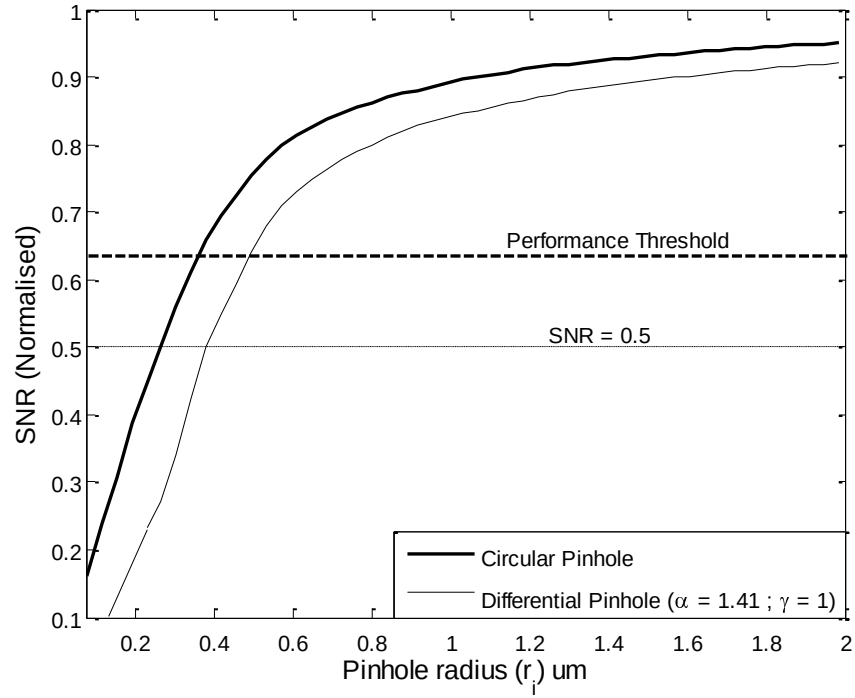


Figure 5.6: SNR Vs pinhole radius (r_p) for a CFM system using a circular pinhole with and a differential pinhole (circular projection of which has $\alpha = 1.41$ and $\gamma = 1$). Here $NA = 0.5$ and $\lambda = 0.5$.

Figure 5.7 plots the absolute values of simulated images of an annular object as imaged by a circular pinhole CFM system (pinhole radius = $0.27 \mu m$) and a differential pinhole (circular projection of which has an outer pinhole radius $0.54 \mu m$, $\alpha = 1.41$ and $\gamma = 1$) CFM system, with radii chosen to give the same SNR in both cases.

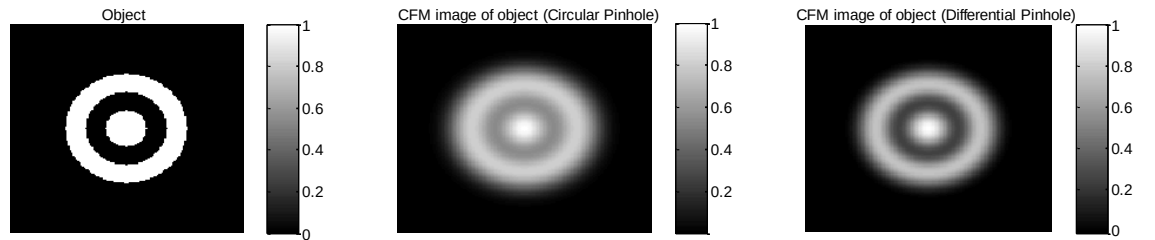


Figure 5.7: Comparing the CFM image of an annular object using a circular pinhole with $r_i = 0.27 \mu m$ and a differential pinhole (circular projection of which has an outer pinhole radius $0.54 \mu m$, $\alpha = 1.41$ and $\gamma = 1$). Object consists of two fluorescent rings of width $0.5 \mu m$ separated by the same distance and the SNR = 0.5.

The images have been generated by convolving the 2D object function with the 2D confocal PSF in the plane of focus. Although absolute values are displayed in figure 5.7, as the total PSF negative dip intensity is small, virtually identical images would be observed by just displaying the positive image intensities. The resolution improvement can be clearly observed here.

In figure 5.8, two planar objects are imaged – one an annular ring structure placed at $z = 0$ and the second a cross placed at $z = 4 \mu\text{m}$ (i.e an inter-object separation of $4 \mu\text{m}$). Both the fluorescent objects have been normalised so that each emits the same mean number of photons for equal uniform illumination. Shown are a series of sectioned images as the focal plane of the illumination is moved between the two object planes. Owing to the tighter PSF mainlobe width and negligible negative sidelobes, the differential pinhole (circular projection of which has an outer pinhole radius $0.54 \mu\text{m}$, $\alpha = 1.41$ and $\gamma = 1$) based system compared to its circular pinhole counterpart (outer pinhole radius $0.27 \mu\text{m}$) shows a greater separation of the two objects e.g. in the third image from the left in the top row the cross is still clearly visible in this image but in the lower image the cross is nearly invisible.

Figure 5.9 and 5.10 further compares the two systems when axial separation between two planar objects is decreased to $2 \mu\text{m}$ at different SNR values. It can be clearly seen that even at such relatively small inter-object separation, up to the SNR threshold on 0.63, the differential pinhole based system produces better quality images than the conventional circular pinhole based counterpart.

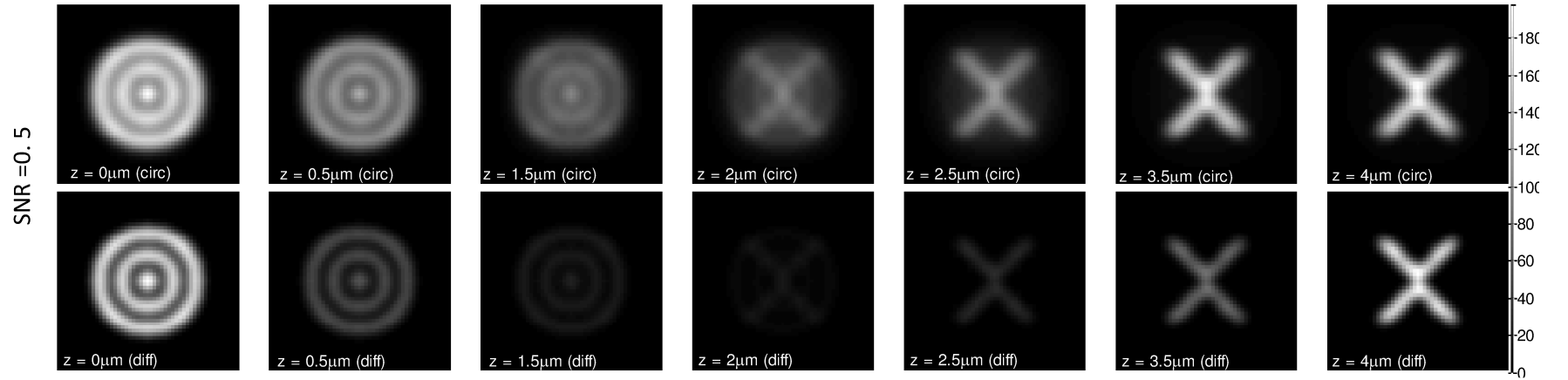
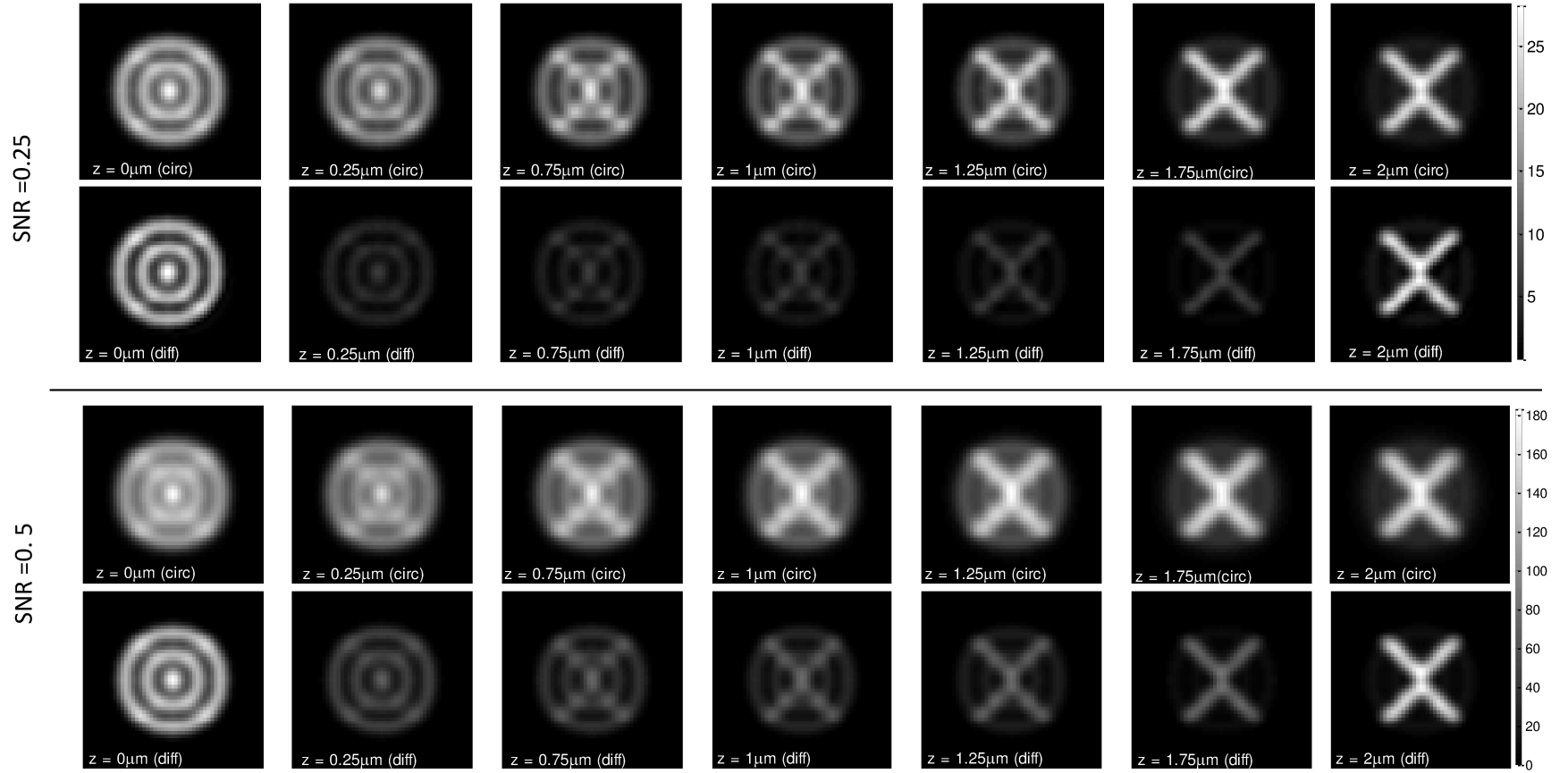


Figure 5.8: CFM images of an annular ring (located at $z = 0$ plane) and a cross shaped object (located at $z = 4 \mu\text{m}$ plane) for an SNR of 0.5 using a circular pinhole (top row) with $r_i = 0.27 \mu\text{m}$ and a differential pinhole (bottom row) (circular projection of which has an outer pinhole radius $0.54 \mu\text{m}$, $\alpha = 1.41$ and $\gamma = 1$). The annular ring object consists of three concentric rings of width $0.3 \mu\text{m}$ separated by $0.3 \mu\text{m}$. The width of lines in the cross shaped object is $0.3 \mu\text{m}$.



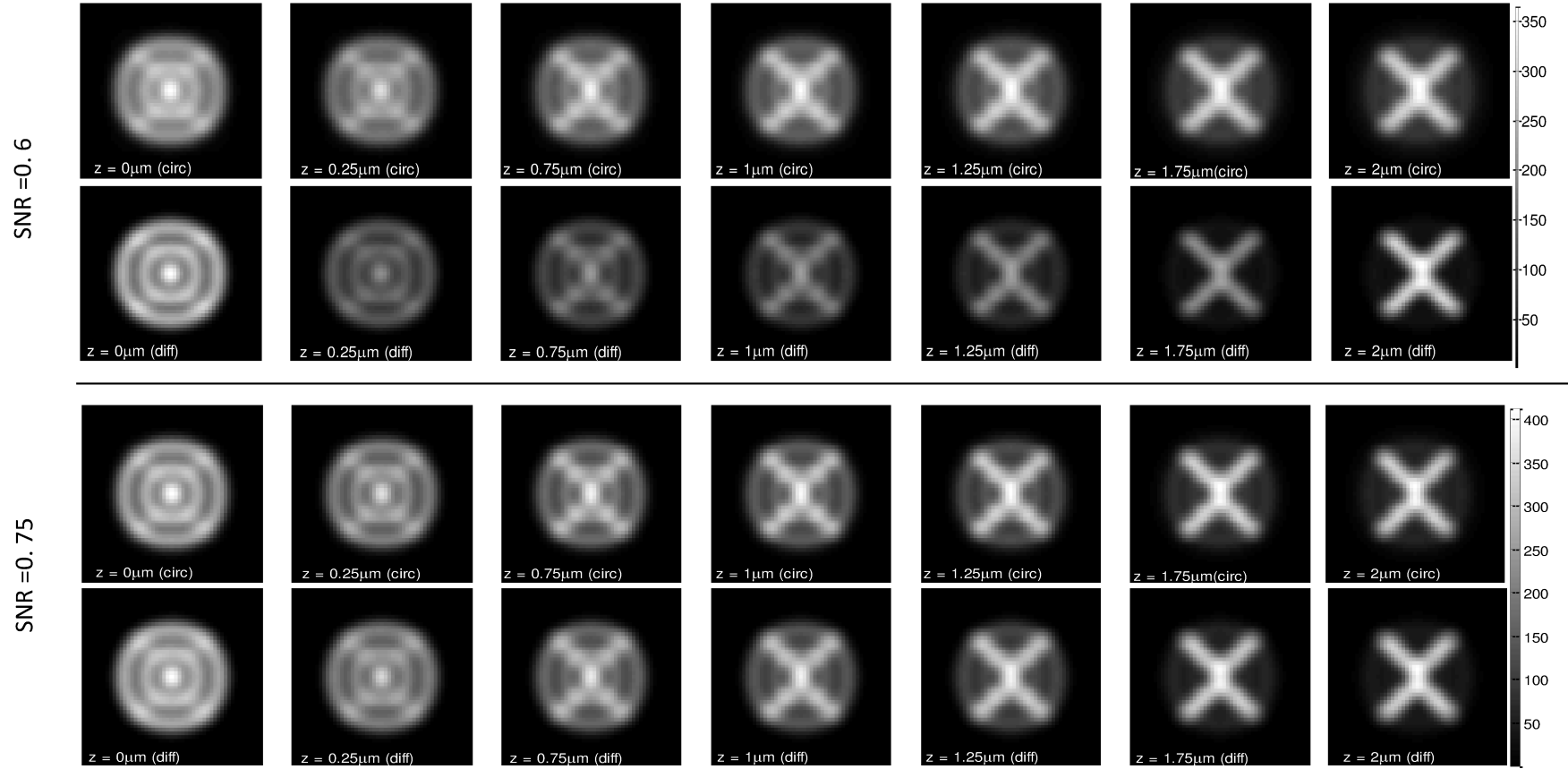


Figure 5.10: CFM images of a ring (located at $z = 0$ plane) and a cross shaped object (located at $z = 2\mu\text{m}$ plane) for an SNR of 0.6 (top panel) and SNR = 0.75 (bottom panel) using a circular pinhole (top row of each panel) and a differential pinhole (bottom row of each panel). Top panel: Circular pinhole with $r_p = 0.4\mu\text{m}$ and the circular projection of the differential pinhole has an outer pinhole radius $0.6\mu\text{m}$, $\alpha = 1.41$ and $\gamma = 1$). Bottom panel: Circular pinhole with $r_p = 0.5\mu\text{m}$ and the circular projection of the differential pinhole has an outer pinhole radius $0.9\mu\text{m}$, $\alpha = 1.41$ and $\gamma = 1$). The width of the ring and the cross shaped object is $0.3\mu\text{m}$.

5.6 Influence of Background noise

In general the noise emanating from stray light (i.e. background) in the measured signal is referred to as background noise. In fluorescence microscopy, background noise is the stray light, arriving at the detector, generated (primarily) by two mechanisms –

- (1) B1: autofluorescence in the cements and coatings of the microscope system (particularly in the objective lens) [13], and
- (2) B2: autofluorescence emanating from fluorophores present outside the resolution volume of the microscope [14]

B1 refers to the stray light that originates from light scattered within the microscope body and from specular reflections in the optical elements. B1 can be assumed to be distributed uniformly over the pinhole detector and is thus proportional to area of the pinhole,

$$B1(v_d) \propto \frac{v_d^2}{4}, \quad (5.3)$$

where v_d is the pinhole radius in optical units. In fluorescence microscopy B1 can be reduced substantially by use of barrier filters [14].

For a thick featureless volume object, the background B2 is the light originating from object fluorescence outside the resolution volume of the objective lens and can be calculated by integrating the intensity at the detector plane $I_D(v, u = 0)$ over the

area of the pinhole. $I_D(v, u)$ is then given by the 3D convolution $\otimes_3$ of the intensity PSF of the illumination lens PSF_1 with that of the collection lens PSF_2 . Thus,

$$I_D(v, u) = PSF_1(v, u) \otimes_3 PSF_2(v, u). \quad (5.4)$$

As shown in figure 5.11, at the detector plane $I_D(u = 0)$ decreases monotonically with distance from the optical axis, decaying rapidly for larger distances as $1/v_d^3$.

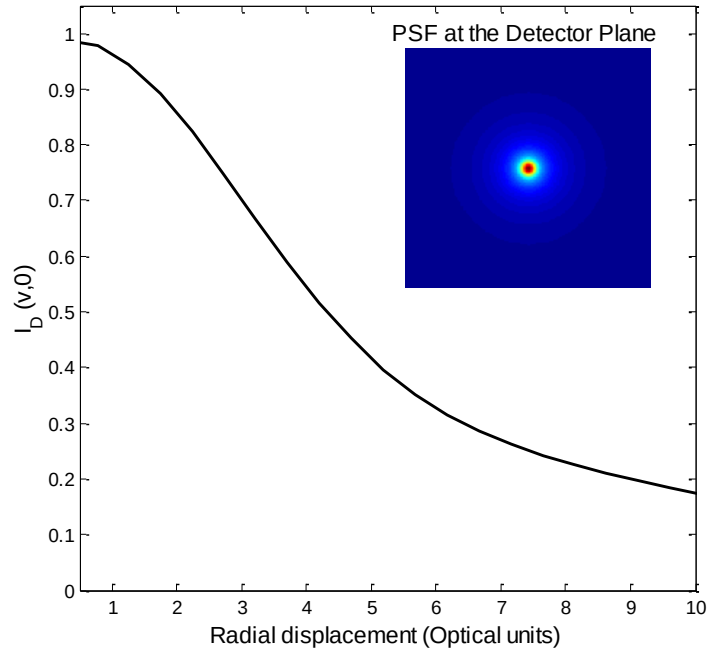


Figure 5.11: Variation of the intensity of fluorescent light arriving at the detector plane when an axially focussed laser beam is used to illuminate a thick featureless volume object. Inset shows the 2D intensity distribution of I_D

For a pinhole detector D, B2 can then be readily calculated as

$$B2(v_d) = \int_D I_D(v, 0) dv. \quad (5.5)$$

For a conventional circular pinhole based CFM system, B_2 as a function of pinhole radius is shown in figure 5.12 (bold line). B_2 here has been normalised such that it has a value of unity for a very large pinhole radius. In the differential pinhole based system each detector receives its own background B_2 . Referring back to figure 5.1(a), let B_{2+} and B_{2-} represent the background detected by detectors D1 and D2 respectively.

For the optimised differential pinhole system ($\alpha = 1.41$ and $\gamma = 1$), proposed in this chapter, it can be seen from figure 5.12 that for very large pinhole radii B_{2+} increases (dashed line) with the pinhole radius while B_{2-} decreases (dotted line). This is because at such radii, almost no light is reflected onto detector D2. At smaller (and more commonly used) pinhole radii (< 1 Airy unit i.e. $0.6 \mu\text{m}$ for system with $\text{NA} = 0.5$ and $\lambda = 0.5 \mu\text{m}$) both B_{2+} and B_{2-} increase linearly with $B_{2+} > B_{2-}$.

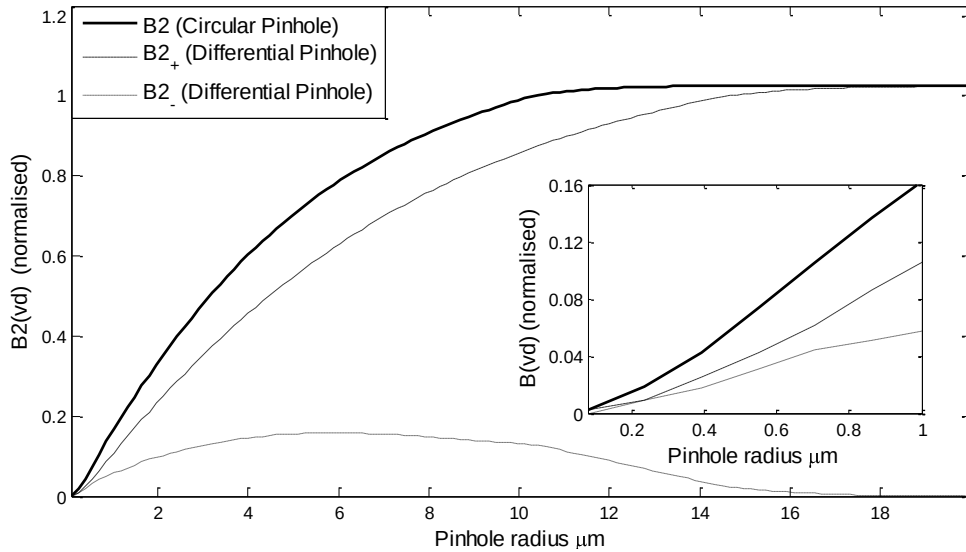


Figure 5.12: Background light from a thick featureless volume object as a function of pinhole radius for a circular pinhole based (bold line) and as a function of inner pinhole radius for a differential pinhole ($\alpha = 1.41$ and $\gamma = 1$) based CFM system. Here $\text{NA} = 0.5$ and $\lambda = 0.5 \mu\text{m}$. The background light variation with pinhole radius for the two systems is also shown in the inset, plotted here for pinhole radii $< 1 \mu\text{m}$.

Now, if a_{B2} represents the magnitude of $B2_+$ and $B2_-$ relative to the CFM signal for a large circular pinhole, a small value representing weak autofluorescence, then the SNR of a differential pinhole system defined earlier in equation 5.3 (neglecting $B1$ and assuming zero dark current noise) can be re-evaluated as,

$$SNR_{diff} = \frac{N_+ - \gamma N_-}{\sqrt{(N_+ + a_{B2}B2_+) + \gamma^2(N_- + a_{B2}B2_-)}} \quad (5.6)$$

Here again N_+ and N_- represent the mean number of signal photons detected by detectors D1 and D2 respectively (but scaled to have a value of unity for a large outer pinhole radius) and γ is the multiplication factor applied to the photon count from the outer annulus. If $a_{B2} = 1$ then the same number photons are detected from a uniform planar object as from background noise.

For the optimised differential pinhole design ($\gamma = 1$ & $\alpha = 1.41$) by plotting SNR_{diff} as a function of pinhole radius (see figure 5.13) it can be observed that as the magnitude of autofluorescence index a_{B2} is increased from a relatively small value of 0.01 (weak fluorescence) to a relatively large value of 10 (strong fluorescence), the SNR curves show maxima at specific pinhole radii (which scales proportional to a_{B2}). For example with $a_{B2} = 0.1$ maximum SNR in a circular pinhole based CFM system can be obtained with a pinhole radius of $1.1\mu\text{m}$ and for an optimised differential pinhole based CFM system at an outer radius of $1.3\mu\text{m}$ (inner radius of $0.92\mu\text{m}$). Operating at these pinhole radii, maximum SNR can be obtained in the respective systems, but this does not result in the best possible resolution.

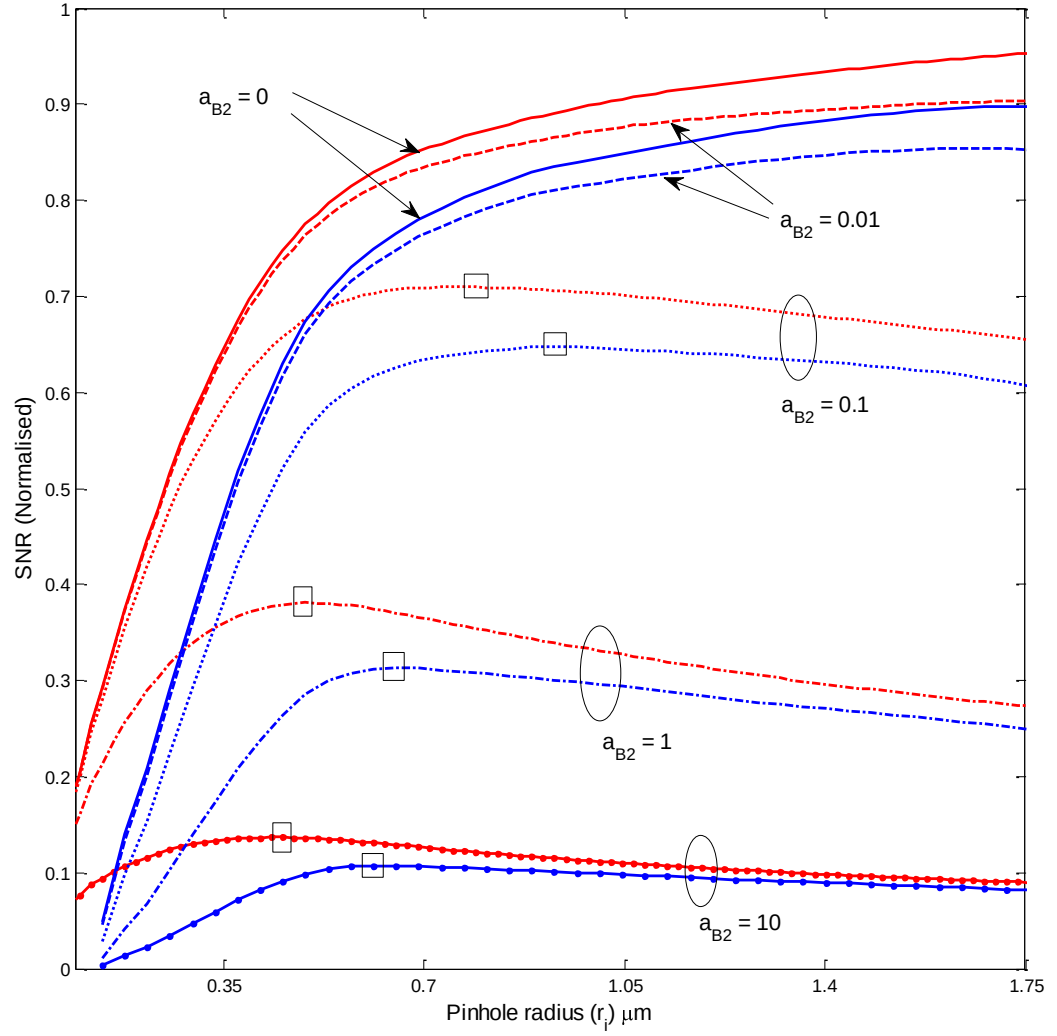


Figure 5.13: Impact of background noise B2 on the SNR performance of a conventional circular based CFM system (curves in red) and an optimised differential pinhole (curves in blue). Here $NA = 0.5$ and $\lambda = 0.5\mu\text{m}$. The $\square$ indicate the pinhole radii (inner radii for the differential pinhole) at which maximum SNR can be achieved using the respective system for a given autofluorescence index a_{B2} .

To better understand the influence of background noise on the performance of a CFM system, the SNR-resolution trade-off characteristic along the lateral and axial direction is plotted in figure 5.14. It can be easily seen (figure 5.14 (a)) that even under strong autofluorescence (large a_{B2}), the optimised differential pinhole system continues to yield a better axial performance than its circular pinhole counterpart over all possible SNR values.

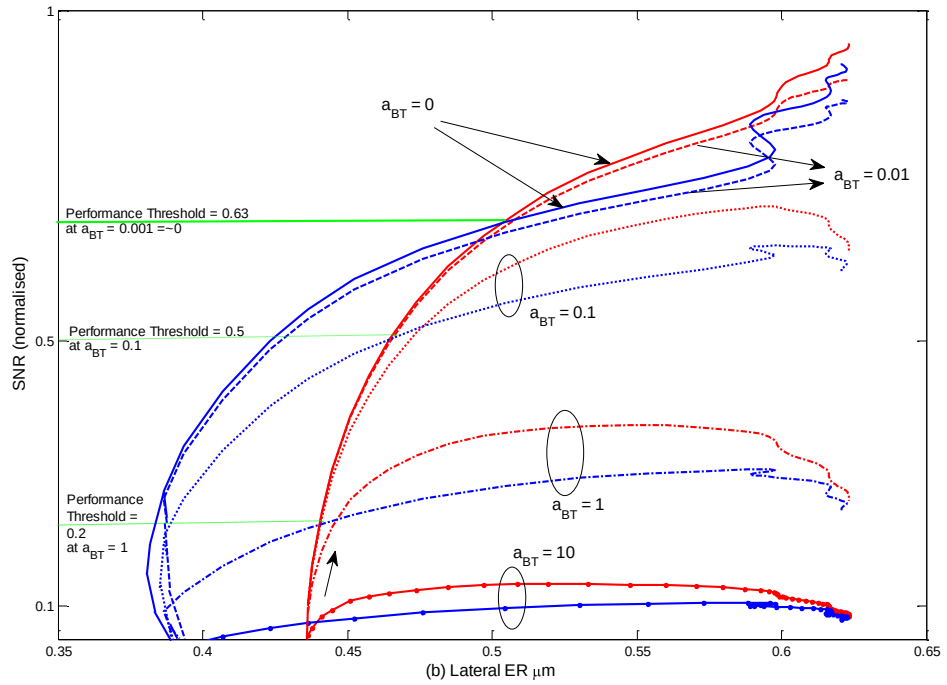
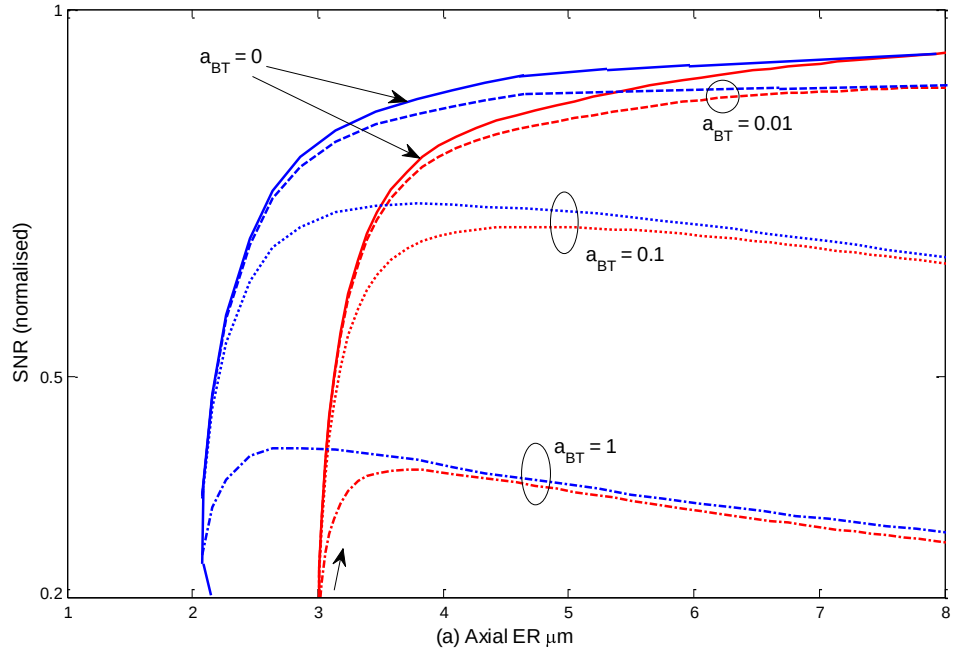


Figure 5.14: SNR-resolution trade-off characteristics of a conventional circular pinhole geometry based CFM system (curves in red) and an optimised differential pinhole based CFM system (curves in blue) for different strengths of background noise for a thick featureless object in the (a) axial and (b) lateral direction. Here $\text{NA} = 0.5$ and $\lambda = 0.5\mu\text{m}$. Arrows at the bottom indicate direction of increasing pinhole radius

In regards to the lateral performance, the optimised differential pinhole based CFM system offers a better trade-off up to certain SNR values marked as ‘performance thresholds’ in figure 5.14(b). Lying below these thresholds are the range of pinhole radii (in the optimised differential pinhole system) that would provide the maximum and simultaneous resolution performance gains along either direction at a given strength of background noise compared to conventional circular pinhole geometry counterpart.

5.7 Using Pupil filters for axial resolution

The use of non-circular pupil filters has been evidenced to provide lateral, axial as well as 3D resolution improvements in confocal microscopes [15-21]. These methods commonly use pupil filters based on annular, multi-annular or leaky annular designs [22-24]. Furthermore, pupil filters based on polarisation filtering (using birefringence) have also been proposed [25-27]. By requiring no significant alteration to the microscope architecture, pupil filters offer a relatively easy and low cost alternative (to the more complex super-resolution techniques such as 4Pi and STED for example) to sharpen the 3D PSF.

It is known that the axial resolution of confocal setups is poor compared to its lateral counterpart. Several attempts have been made to improve axial resolution, one such being 4Pi microscopy [28] which substantially narrows the axial response by interference of two opposing tightly focussed wave fronts. Subtractive imaging offers an alternative approach to improving axial resolution and has been coupled previously with different pupil filters to extend the axial resolution even further. For

example Martinez-Corral et al [29] have demonstrated a 25% increase in axial resolution using a zero-focus complex transmittance filter [30] in one of the collector paths of a two-pinhole subtractive imaging configuration. The optimised differential pinhole based CFM system, based on the subtractive imaging approach, proposed in the previous section was shown to be capable of yielding simultaneous and maximum performance gains (up to an SNR of 0.63) in both the lateral axial directions using clear pupils in both the illumination and collection paths. It is interesting to examine the potential of using existing pupil filter techniques to further increase the axial resolution of the proposed method.

A very recent publication by Ye Ma et al [31] demonstrated the use of a conjugate set of so called “fan” shaped pupil filters to considerably improve the axial resolution of a confocal microscope using finite sized pinholes. Fan shaped filters were shown to offer better axial resolution in comparison to circular apertures and some of the other previously used pupil filters e.g. D-shaped (segments of a circle) [32] and annular shaped (concentric rings) [33]. The fan shaped pupils however demonstrated no advantage in the lateral direction (over clear pupils). However, the improved axial resolution makes the method suitable for particle tracking or axial displacement measurement wherein better axial response sensitivity (rather than lateral resolution) is required [31].

This section will investigate the potential of using fan shaped filters in an optimised differential pinhole based CFM system.

5.7.1 Fan shaped pupils

If x_p and y_p are the normalised (normalised by the pupil radius a) coordinates on the pupil plane, then the pupil function of a fan shaped aperture can be represented mathematically as [31],

$$P_{fan}(x_p, y_p) = \begin{cases} 1, & x_p^2 + y_p^2 \leq 1 \text{ and } x_p \geq d_1 \text{ and } y_p \geq d_2 \\ 0, & \text{otherwise} \end{cases} \quad (5.7)$$

Here d_1 and d_2 are the distance parameters that control the distance from the centre of the circle to the edge of the aperture. Ye Ma et al [31] find that maximum axial resolution can be obtained with $d_1 = d_2 = 0.2$, and hence the same shall be examined in the context of the optimised differential pinhole based CFM system. Figure 5.15 shows the illumination and collection apertures and the infocus PSF of a CFM system using clear pupils and fan shaped pupils ($d_1 = d_2 = 0.2$).

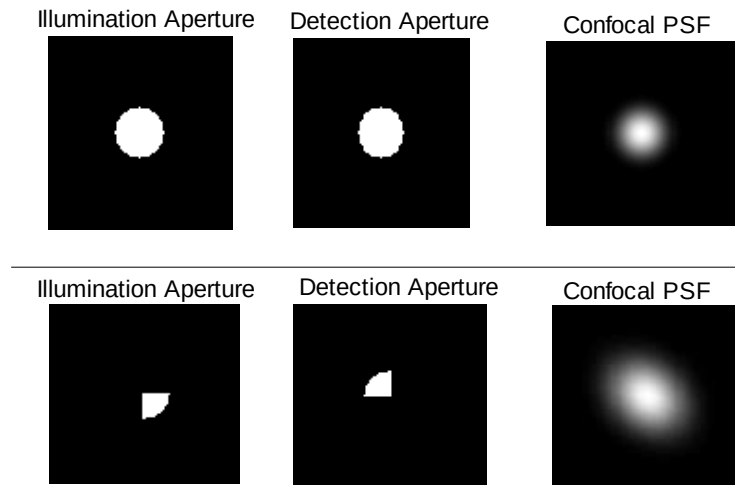


Figure 5.15: Two different sets of illumination/collection apertures for a conventional circular pinhole based CFM system and the resulting confocal PSF. Top row – circular clear pupils. Bottom row – Fan shaped pupils $d_1 = d_2 = 0.2$. Here $NA = 0.5$ and $\lambda = 0.5\mu\text{m}$ and pinhole has a radius of $0.6\mu\text{m}$.

It should be apparent that in the case of fan shaped pupils, the elliptical elongation of the confocal infocus PSF results in an asymmetrical and lower lateral resolution in comparison to its circular pupil counterpart. Figure 5.16(b) shows the infocus PSF intensity measured along the major and minor axis (lateral direction) when the pinhole radius is $0.6 \mu\text{m}$ (with $\text{NA} = 0.5$ and $\lambda = 0.5 \mu\text{m}$). There is a substantial broadening ($\sim 63\%$) of the PSF when compared to the PSF using clear circular pupils.

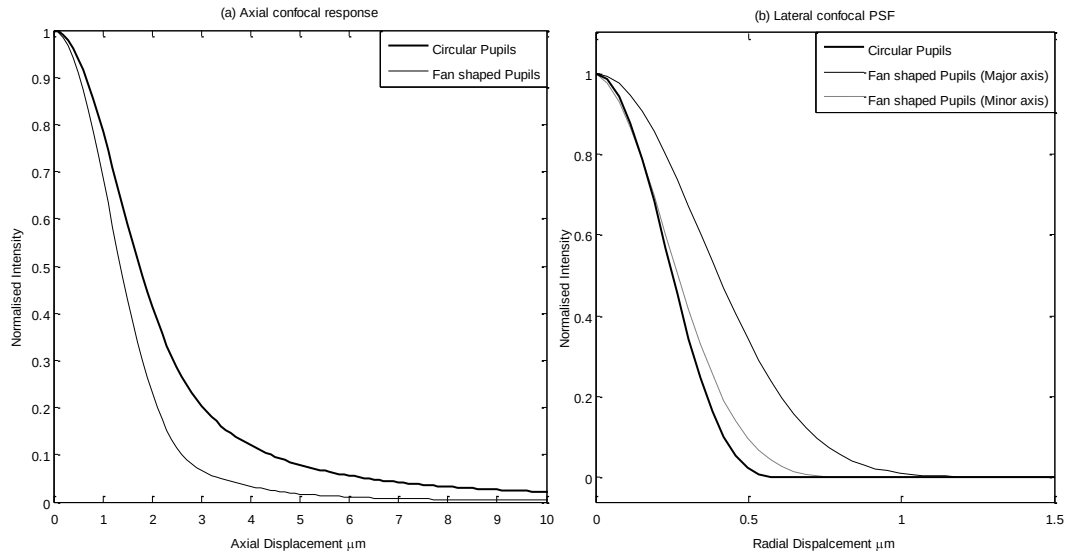


Figure 5.16: Axial response to a uniform planar object of a conventional circular pinhole based CFM system using clear circular pupils and fan shaped pupils. Here $\text{NA} = 0.5$ and $\lambda = 0.5 \mu\text{m}$ and pinhole has a radius of $0.6 \mu\text{m}$. The FWHM of the axial confocal response reduces by about 22% using fan shaped pupils. Inset shows the lateral response of the same system using the two pupil types. Lateral FWHM increases by about 12% along the minor axis and by 63% along the major axis using fan shaped pupils.

At the same pinhole radius however, there is a considerable reduction ($\sim 22\%$) in the axial response (see figure 5.16(a)). As demonstrated earlier in this chapter, the optimised differential pinhole is also capable of offering axial as well as lateral resolution gains. By coupling the ODP with fan shaped pupils, further axial resolution gains can be expected with the potential of mitigating the loss of lateral

resolution experience from the use of fan shaped pupils. This is investigated in the next section.

5.7.2 Differential pinhole based CFM system using fan shaped pupils

Consider a conventional CFM setup (figure 3.11) with fan shaped apertures shown in figure 5.15 (bottom panel) and coupled with differential pinhole arrangement described in figure 5.1. For the same pinhole radius of $0.6 \mu\text{m}$ with $\text{NA} = 0.5$ and $\lambda = 0.5 \mu\text{m}$ and using the optimised differential pinhole (ODP), both the lateral and axial CFM responses shrink substantially as shown in figure 5.17 and 5.18, respectively, but result in some undesired negative sidelobes.

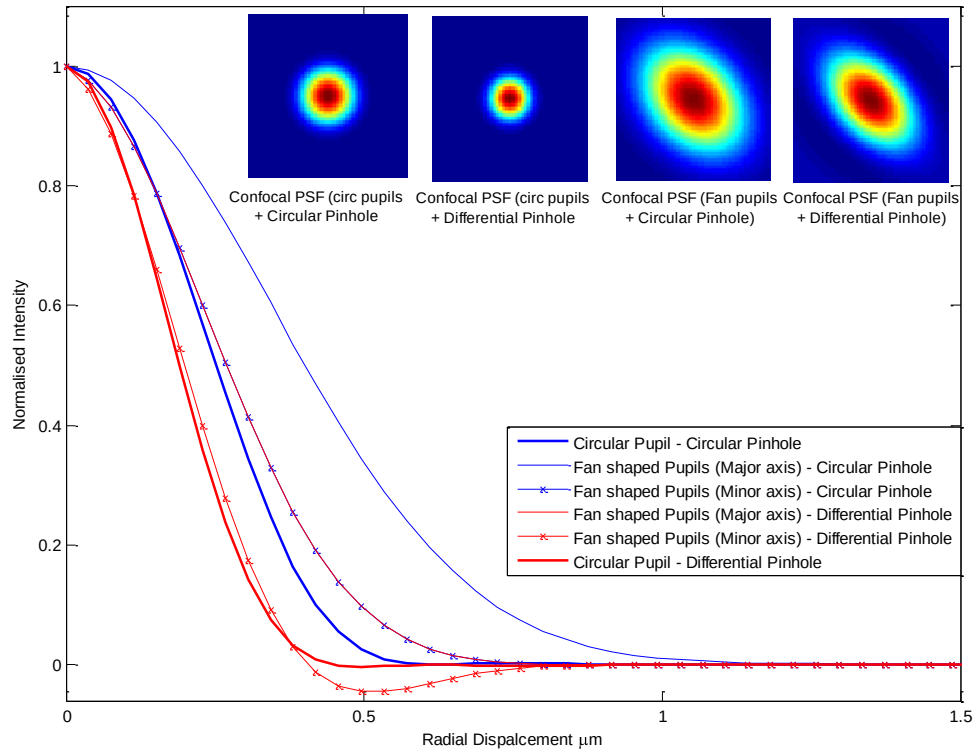


Figure 5.17: Lateral response of a CFM system with a pinhole radius of $0.6 \mu\text{m}$ (inner radius in the case of a differential pinhole). Here $\text{NA} = 0.5$ and $\lambda = 0.5 \mu\text{m}$. Inset shows the infocus confocal PSFs for four different permutations of pinhole and pupil combinations.

Along the lateral direction, the broadening of PSF resulting from the use of fan shaped filters in conventional circular pinhole CFM system is mitigated along both major and minor axis using the differential pinhole. Although the loss in lateral resolution is notably reduced, it can be inferred from the infocus responses (see inset of figure 5.17) that the fan shaped filter based ODP CFM system offers lower resolution when compared to its circular pupil ODP CFM counterpart.

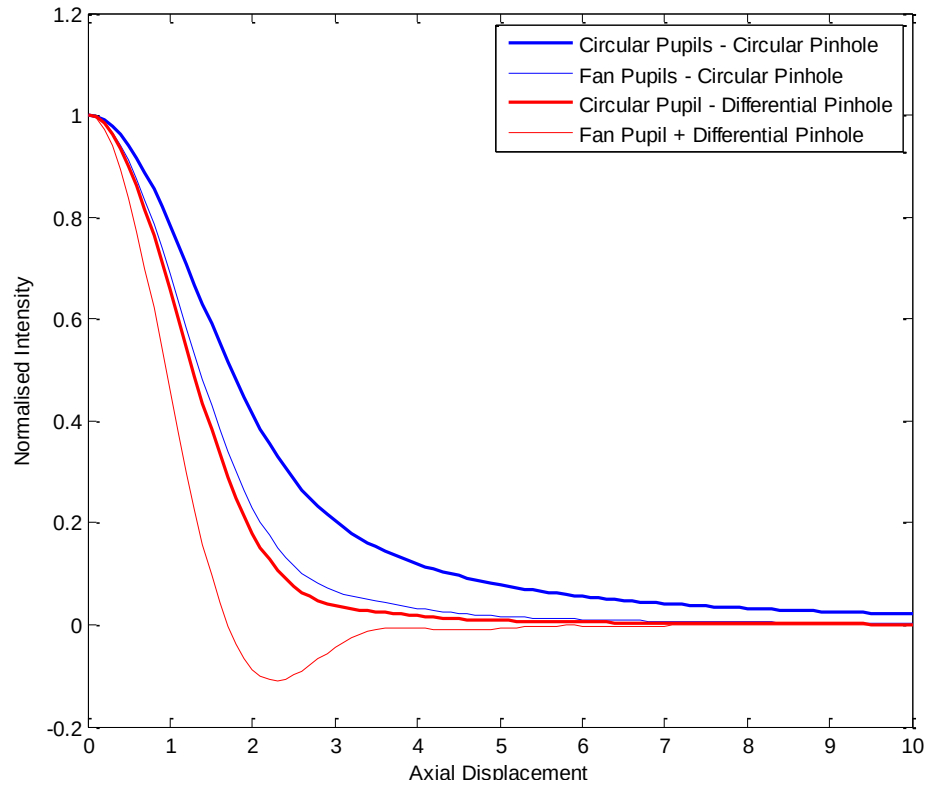


Figure 5.18: Axial response of a CFM system with a pinhole radius of $0.6 \mu\text{m}$ (inner radius in the case of a differential pinhole) using different pinhole and pupil combinations. Here $\text{NA} = 0.5$ and $\lambda = 0.5 \mu\text{m}$.

In the axial direction, the response shrinks substantially (figure 5.18) using fan shaped filters and ODP when compared to its circular pupil counterpart, but results in larger negative sidelobes. The conventional FWHM resolution metric fails to account for sidelobes and thus the ER metric, shown earlier in the chapter to be able

to account for such negative sidelobes, is used here. Using the equivalent radius (ER) metric, the SNR-axial resolution trade-off curve for the fan shaped pupil filter CFM system is plotted in figure 5.19 (a) (curve in cyan).

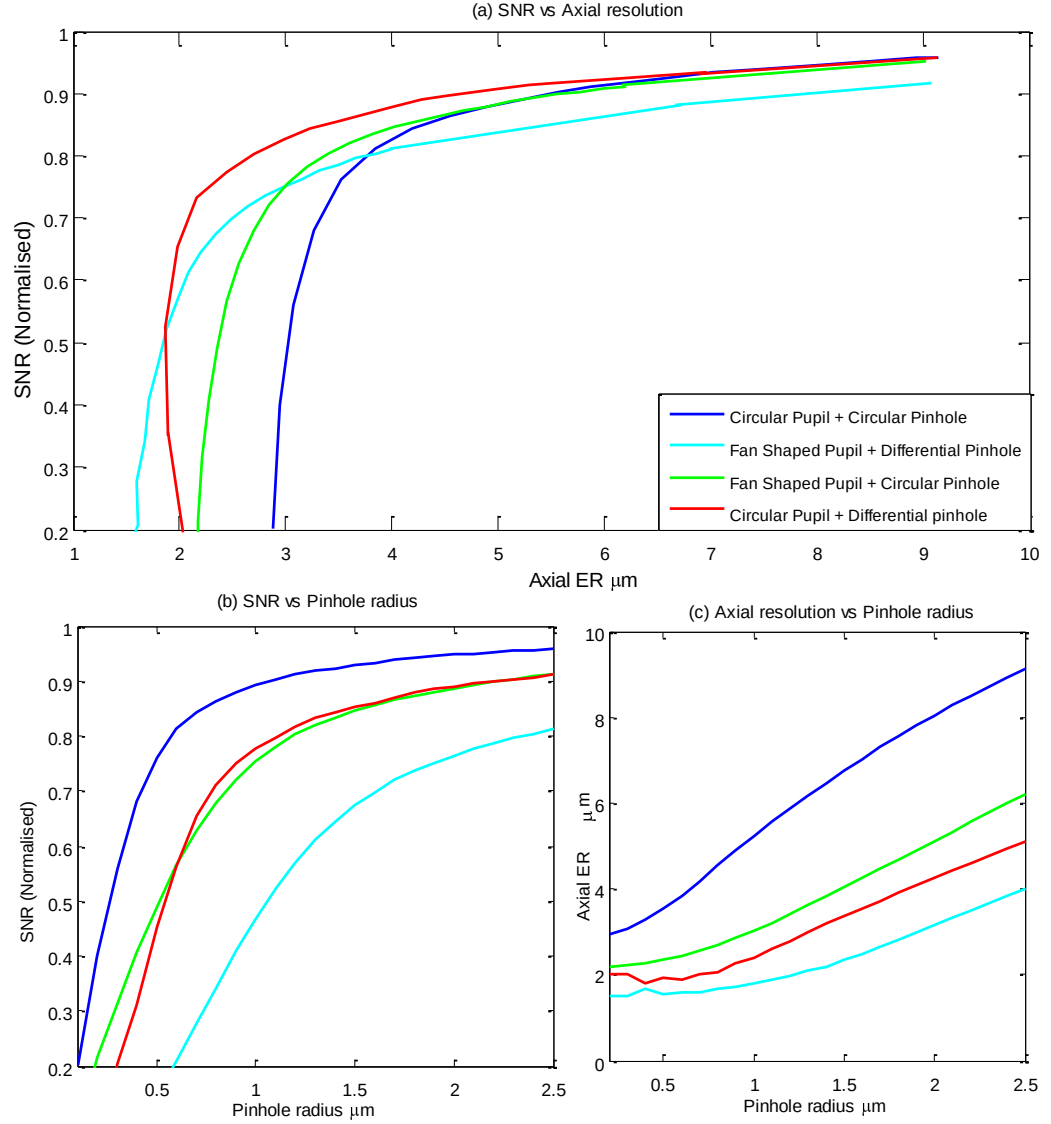


Figure 5.19: (a) SNR versus axial resolution (ER based) trade-off characteristics for two different pinhole (circular and optimised differential pinhole) based CFM system using two different pupil types (circular clear pupils and fan shaped pupils). (b) SNR as a function of pinhole radius (inner pinhole radius for the differential pinhole) for different pinhole and pupil types. (c) ER based axial resolution as a function of pinhole radius for different pinhole and pupil types. Here $\text{NA} = 0.5$ and $\lambda = 0.5\mu\text{m}$.

The ODP based CFM system using circular pupils completely outperforms the conventional circular-pinhole based CFM system using fan shaped pupils. This is because although both the systems offer nearly the same SNR (see figure 5.19 (b)), for all pinhole radii, the circular pupil ODP based CFM system offers relatively better axial resolution (see figure 5.19(c)).

Coupling an ODP with fan shaped pupils results in substantial degradation in the SNR (figure 5.19(b)), but substantial improvement in the axial resolution due to tightening of the axial response. For pinhole radii up to 1 μm , fan shaped pupils further improve the axial performance of an ODP based CFM system. Examining figure 5.19(a), ODP based CFM system using fan shaped pupils can offer considerable improvement up to an SNR of 0.5. It should be noted though that the lateral resolution of this system is poorer than the conventional circular pinhole based clear pupil CFM system.

5.8 Chapter Summary

- An optimum profile for the circular projection of an elliptically shaped differential pinhole has been deduced through the introduction of a new resolution metric, equivalent radius, which overcomes the inability of the conventional FWHM to take into account sidelobes in the PSF.
- For $\text{SNR} < 0.63$ it has been shown that simultaneous resolution gains over a conventional circular pinhole geometry based CFM system, along both the lateral and axial directions can be obtained by setting the parameters $\alpha = 1.41$ and $\gamma = 1$ for the differential pinhole.

- In regard to the SNR-axial resolution trade-off, the differential pinhole system outperforms the conventional system, with the optimum parameter set, for all SNRs values.
- Requiring no significant modification to the CFM setup and using fast point detectors, the now optimised differential pinhole detection based CFM system offers significant 3D resolution gains at $\text{SNR} < 0.63$. The results show that higher quality images are produced when employing this pinhole geometry compared to the conventional circular pinhole geometry.
- Even under the presence of strong autofluorescence, the optimised differential pinhole based CFM system outperforms the conventional CFM system for all SNR values in the axial direction.
- Alternative pupil filters, such as the recently proposed fan shaped pupil filter, which offers axial resolution improvement in confocal microscopy, when used in an optimised differential pinhole based CFM system, further improves the axial performance of the differential pinhole based CFM system up to an SNR of 0.5.

5.9 References

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CHAPTER 6

Performance improvement using an array detector and Maximum Likelihood Processing

In this chapter, an alternative detection arrangement is examined in which the entire detection/image plane is recorded using an array detector rather than a pinhole detector. Using this recorded data an attempt is then made to recover the object from the whole set of recorded photon array data; to do this maximum-likelihood estimation (MLE) has been applied. The recovered object estimates are shown (through computer simulation) to have good resolution, image sectioning and signal-to-noise ratio compared with conventional pinhole CFM images.

6.1 Recording photons using array based detectors

Measurement imperfections and inherent imaging properties of fluorescence microscopes distort the 3D images they generate, limiting the maximum obtainable resolution. Fluorescence microscopes experience two types of distortions – a deterministic blurring by the point spread function (PSF) (see figure 6.1; adapted from [1]) and a stochastic disturbance (noise; see flow diagram in figure 6.1 [2]) due to the limited number of photons emitted and detected. As demonstrated in the preceding chapters, in a confocal fluorescence microscope [3-5] the employment of a pinhole enhances the resolution and image sectioning ability. It achieves this by rejecting light that does not pass through the detection pinhole; thus this detection

arrangement further limits the number of photons that are collected with a subsequent impact on the signal to-noise ratio (SNR).

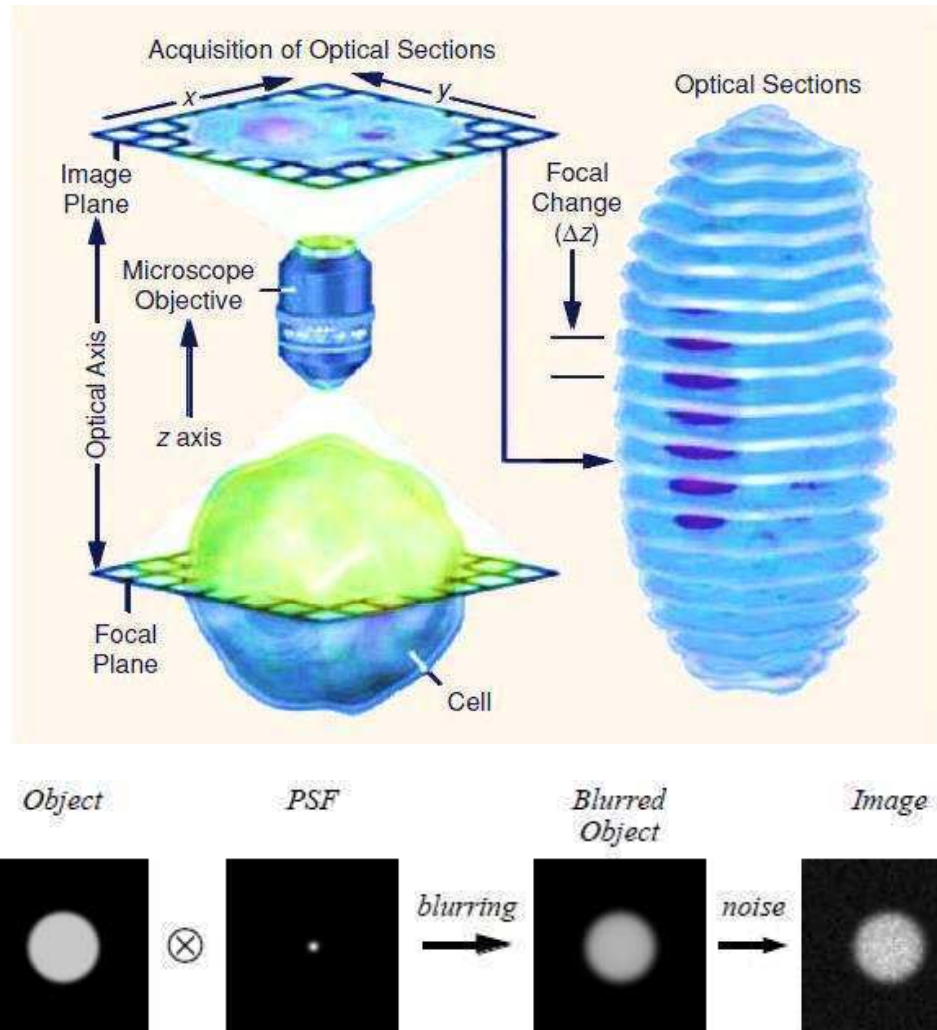
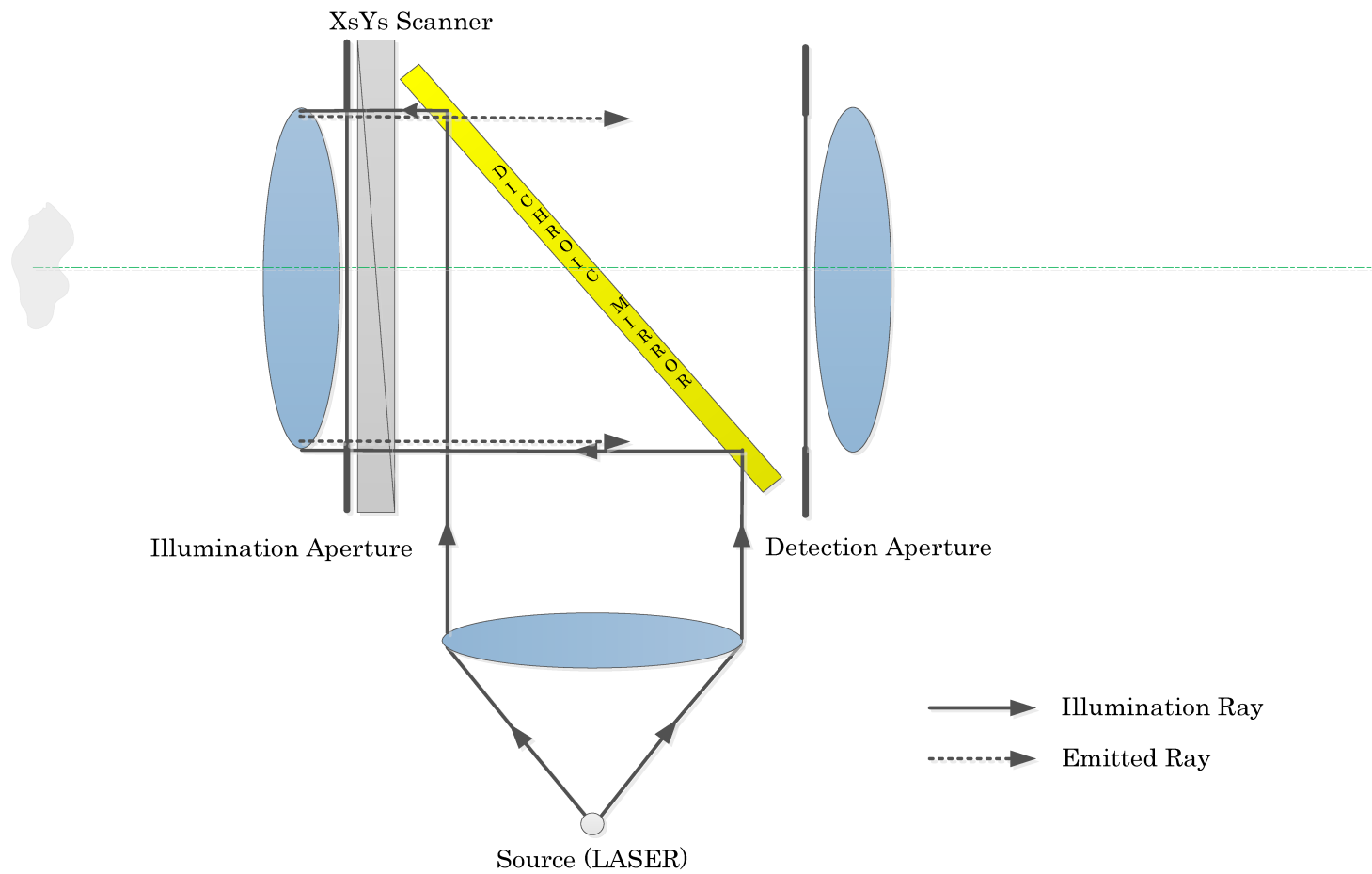


Figure 6.1: Top row - 3D image acquisition of a cell captured by a fluorescence microscope (adapted from [1]). Reflected fluorescent light from each scan of a given focal plane slice forms a 2D image. Moving through focus, several images are collected and then stacked to form the final 3D reconstruction of the cell. 3D image appears elongated along the axial direction but maintains its spherical shape in the lateral. Bottom row – Simple flow-diagram [2] of the image formation in FM. The $\otimes$ represents a convolution operator.

By using novel detection schemes such as the one described in chapter 5 (based on subtractive imaging technique [6-11]) or using alternative pinhole geometries in conjunction with pixel array detectors (chapter 4) it is possible to improve the 3D

resolution of a CFM system. Although these techniques improve the SNR-resolution trade-off; both approaches result in a smaller SNR in comparison to existing (circular pinhole based) CFM systems. An alternative approach is, at each illumination scan position, to record not just the number of photons that pass within the radius of a pinhole but rather to record using a detector array all the photons in the confocal plane. A schematic of one possible optical arrangement to implement this type of data collection is shown in figure 6.2. Clearly this will mean that more photons will be detected, compared with a pinhole detection arrangement, with the potential that more useful information might be extracted from this expanded data set.

The obvious problem of this approach is that in conventional CFM a display of the recorded photon count at each scan position gives a usable image; whereas in the proposed approach some further processing will be necessary to produce an image. Longer data acquisition and image processing times although undesirable are often traded off for better image quality in medical imaging. The idea of inverting data collected from an array detector in a CFM was first proposed nearly thirty years ago by Sheppard [12] and Bertero [13]. Sheppard proposed forming multiple images, with each image formed by using a single detector pixel, treated as a shifted pinhole. After applying suitable shifts to each image to allow for the offset induced by using a displaced pinhole they could be summed to form a composite image. Bertero proposed using a truncated singular function expansion method to obtain a reconstructed image.



However neither approach seems to have been implemented commercially, possibly due to the noise performance of the methods. Recently, Zeiss have produced a commercial CFM instrument, ‘Airyscan’ employing an array detector with 32 elements in place of a single pinhole [14]. This instrument uses a similar approach to the Sheppard proposal, but the image formed by each detector in the array is deconvolved, using a Weiner filter approach, before weighted summing.

6.2 Image reconstruction in optical microscopy

Blurring of an image by the PSF of an optical microscope hampers the quantitative analysis of the image by decreasing the accuracy of the measurements made. In fluorescence microscopy, blurring can be classified as being of two types:

1. **Deterministic** – due to the diffraction of fluorescent light by the system optics, and
2. **Stochastic** – distortions (noise) in the image from various sources (shot noise, dark current, background noise etc.).

Image reconstruction refers to techniques that attempt to mitigate the blurring with the goal to reconstruct the original object from the acquired image. Figure 6.3 shows the general procedure for image reconstruction of a sample object whose image is blurred deterministically by the system PSF and stochastically by Poissonian noise. All methods applied for image reconstruction, outside blind deconvolution, require priori knowledge of the PSF. Blind deconvolution techniques aim to reconstruct the original sample from the image and generate a PSF as a by-product [15]; these

techniques tend to be used when information on the image degradation is very limited.

Deterministic distortions can be removed using a simple inverse filtering approach [16] wherein the Fourier transform of the image is divided by the system optical transfer function. This however results in intolerable amplification of noise (see figure 6.3; adapted from [2]) and thus more regularisation to the restoration procedure is called for.

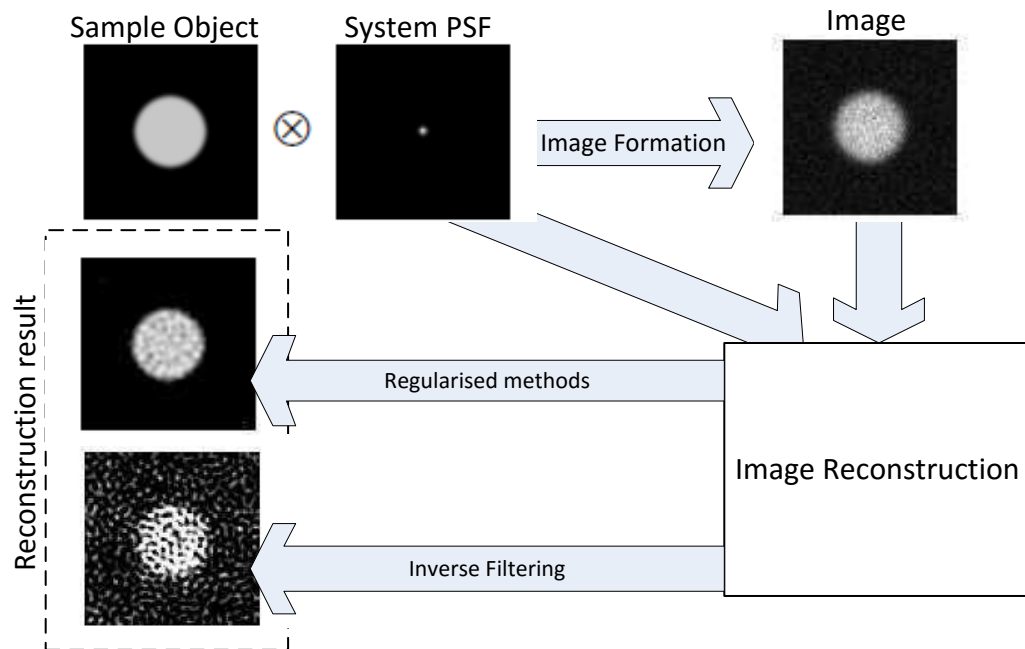


Figure 6.3: Reconstruction applied to the image of a spherical planar object using inverse-filtering resulting in high amplification of noise. Significantly improved reconstruction result is achieved when information of the system PSF is used by the reconstruction algorithm (adapted from [2])

The Weiner and Tikhonov-Miller (TM) filter methods address this by suppressing the noise dominated frequencies while restoring the object dominated frequencies [17].

A Weiner filter minimises the mean square error between the restored estimate and the original image. The TM filter minimises the Tikhonov polynomial which is the

squared difference of the original image and the blurred estimate regularised by the Tikhonov energy band. It assumes that the original image is disturbed by additive Gaussian noise. Both these filter techniques apply linear operations on the recorded image thus becoming prone to ringing artefacts in the restored image brought about by even the smallest PSF data errors. They also sometimes estimate negative intensities; unacceptable in a fluorescent image since intensities represent photons of which only a positive number can exist.

At the cost of increased computational complexity non-linear iterative algorithms include additional information (e.g. object smoothness, estimate non-negativity) to alleviate the aforementioned problems. The two most widely used are the Jansen-van Cittert (JVC) method of repeated convolution with alterations by Agard et al [18] and the least-squares Carrington algorithm [19]. The JVC algorithm although it converges rapidly is incapable of compensating low SNR images. The Carrington technique on the other hand enforces sample estimate non-negativity but converges slowly, thus demanding longer run-times to offer a good object restoration [20].

The methods above are all based on assuming an additive Gaussian noise model (suited for high SNR images) and lack a direct noise reduction strategy [3]. Historically, Shepp and Vardi [21] and Geman and Geman [22] were the first to explore image restoration based on the statistical property of the noise. Implementing more subtle noise strategies and simple regularisations, these statistical methods have proven to be more effective in restoring 3D images with high noise [23].

One widely used method is the expectation-maximisation-maximum-likelihood (EM-ML) method. A non-linear technique based on Poisson noise statistics [24], EM-ML method offers a better trade-off between noise robustness [25, 26] and resolution improvement [27, 28] over deconvolution methods. The algorithm calculates iteratively the maximum likelihood estimator under Poissonian noise which is regarded as the chief limiting noise in fluorescence microscopy [3]. A more detailed comparison of these restoration techniques can be found in [29].

6.3 Image reconstruction using Maximum Likelihood processing

A wide variety of image restoration techniques [3, 15-36] have been shown to effectively reduce image distortions whilst offering an improved signal-to-noise ratio. Although, primarily these techniques have been applied to restoring conventional (wide-field) microscope images, they have proven their ability to enhance images in other types of microscopes such as confocal [26, 33, 37], multiphoton fluorescence microscopes [34, 35] and SIM [38] .

Of the various approaches that have been suggested, the maximum-likelihood estimation (MLE) also known as the expectation maximisation (EM) has some advantages that suggest it as a suitable method to apply to the data-processing problem outlined above. It is a non-linear technique based on Poissonian noise statistics [24], which is regarded as the chief limiting noise in fluorescence microscopy [3]. The algorithm also has an intrinsic non-negativity constraint, which is compatible with a fluorescent object/image. The method is numerically stable, finding a solution through multiplicative iterative changes to the object estimate;

although the slow convergence can be seen as a drawback of the method [24]. MLE has been applied previously to restoring conventional pinhole CFM images [37] but not to treating data from a detector array arrangement such as that of figure 6.1.

The next section will outline the derivation of an MLE algorithm to attempt to restore a maximum-likelihood estimate from a data set consisting of an array of photon counts recorded at each scan illumination position.

6.4 MLE based 3D object reconstruction applied to CFM

Consider a 3D object with fluorophore concentration O . From a point (x_o, y_o, z_o) in the object, the intensity of light emitted upon being illuminated by a focussed beam of light directed to a scan position (x_s, y_s, z_s) is given by,

$$E(x_o, y_o, z_o; x_s, y_s, z_s) = O(x_o, y_o, z_o) P^1(x_o - x_s, y_o - y_s, z_o - z_s), \quad (6.1)$$

where P^1 is the 3D point-spread function of the illumination lens. This emitted (fluorescent) light is then imaged at (x_i, y_i) by the detection lens (3D point-spread function P^2). Assuming that the scanning (both in the lateral and axial directions) is corrected by the scanning optics on the return path so that the centre of the illuminated region is imaged back to the centre of the detection array, then the intensity (mean photon count) arriving at the detection plane may be expressed as,

$$\begin{aligned} N(x_i, y_i; x_s, y_s, z_s) &= \int_{z_o} \int_{x_o} \int_{y_o} O(x_o, y_o, z_o) P^1(x_o - x_s, y_o - y_s, z_o - z_s) P^2(x_i \\ &\quad - x_o + x_s, y_i - y_o + y_s, -z_o + z_s) dx_o dy_o dz_o, \end{aligned} \quad (6.2)$$

where x_i and y_i are the coordinates of the detection plane and z_i is equal to zero as the detector plane is in focus. For a discrete detector array and where the object is regarded as a discretely sampled function, the above integral may be expressed in a compact form as,

$$N_{i,s} = \sum_o O_o P_{o-s}^1 P_{i-o+s}^2, \quad (6.3)$$

where O_o represents $O(x_o, y_o, z_o)$, P_{o-s}^1 represents $P^1(x_o - x_s, y_o - y_s, z_o - z_s)$ etc. and the summation over o represents a summation over all three dimensions. Let $n_{i,s}$ represent the data recorded in the scan of an object i.e. the number of photons recorded by the pixel at (x_i, y_i) due to illumination positioned at (x_s, y_s, z_s) . Because the probability of detecting $n_{i,s}$ photons is governed by a Poissonian distribution controlled by the mean number of photons; for an assumed object distribution O_o ; the probability of recording the photon count in the i^{th} pixel, for scan position s , is given by,

$$Pr(n_{i,s}; O_o) = \frac{N_{i,s}^{n_{i,s}} e^{-N_{i,s}}}{n_{i,s}!}. \quad (6.4)$$

Now the probability $Pr_T(n; O_o)$ to obtain the entire data set (i.e. the full photon distribution at the image plane for each scan position of the beam within the object space) is given by the product of all individual probabilities $Pr(n_{i,s}; O_o)$. Thus,

$$Pr_T(n; O_o) = \prod_{i,s} Pr(n_{i,s}; O_o) = \prod_{i,s} \frac{N_{i,s}^{n_{i,s}} e^{-N_{i,s}}}{n_{i,s}!}, \quad (6.5)$$

where the product is over the three dimensions of the scan positions and the two dimensions of the detector array. The principle of maximum likelihood is to find the object form O_o that maximises the probability of producing the data set $n_{i,s}$.

To find the maximum of this probability requires performing a differentiation and to facilitate this it is convenient to take the logarithm of this probability. Taking the natural logarithm, the product (equation 6.5) is converted into a summation, referred to as the log likelihood function $LL(n: O_o)$. Thus,

$$LL(n: O_o) = \sum_{i,s} n_{i,s} \ln(N_{i,s}) - N_{i,s} - \ln(n_{i,s}!). \quad (6.6)$$

This step can be justified because logarithm is a monotonic function for positive arguments; so the maximum of the probability in equation (6.5) will coincide with the maximum of the log-likelihood in equation (6.6) and the object function O_o which maximises equation (6.6) will be the maximum likelihood estimate. Maximising the above function w.r.t. to the assumed object shape O_o is achieved by setting the differential to zero,

$$\frac{\partial LL(n: O_o)}{\partial O_o} = 0. \quad (6.7)$$

Substituting equation (6.6) in the numerator of (6.7), performing the differentiation and rearranging, gives,

$$\frac{\sum_{i,s} \frac{n_{i,s} P_{o-s}^1 P_{i-o+s}^2}{N_{i,s}}}{\sum_{i,s} P_{o-s}^1 P_{i-o+s}^2} = 1. \quad (6.8)$$

Using this relationship, it is possible to define an operator D, defined by

$$D(O_o) = O_o \cdot \frac{\sum_{i,s} \frac{n_{i,s} P_{o-s}^1 P_{i-o+s}^2}{N_{i,s}}}{\sum_{i,s} P_{o-s}^1 P_{i-o+s}^2}, \quad (6.9)$$

and to then to write (for this operator),

$$O_o = D(O_o). \quad (6.10)$$

From this equation, it may be seen that the required solution for O_o is a fixed point of the operator D. Where such a fixed point exists, it may be shown [21, 39, 40], that an iterative algorithm of the form,

$$O_o^{new} = D(O_o^{old}), \quad (6.11)$$

should converge to that fixed point, i.e. in this case the maximum-likelihood solution. Finally by separating the order of the summations in the operator the iterative algorithm may be expressed as,

$$O_o^{e(new)} = O_o^{e(old)} \times \frac{\sum_s P_{o-s}^1 \sum_i \frac{n_{i,s} P_{i-o+s}^2}{N_{i,s}}}{\sum_s P_{o-s}^1 \sum_i P_{i-o+s}^2}. \quad (6.12)$$

It may be seen that the summations over i and s represent convolutions in 2 and 3 dimensions respectively.

The iteration is normally started with a uniform 3D object estimate and is terminated according to some specified stopping criterion. The issue of stopping MLE iterative algorithms has been studied in the field of positron-emission tomography and a number of statistical criteria have been proposed [41, 42]. For the

results shown in the next section a simple criterion based on comparing the expected noise on the data is used. This involves constructing, from the data, a 3D type-I image, I_{data} ; this is achieved by simply adding together all the data arrays $n_{i,s}$ with each one positioned at its 3D scan position. Following each iteration, the current MLE estimate of the object is used to calculate an estimate of this type-I image, I_{MLE} . The algorithm is judged to have converged, when the variance of these two images ceases to reduce significantly. This is assessed by calculating the following χ^2 statistic,

$$\chi^2 = \frac{\sum (I_{data} - I_{MLE})^2}{\sum I_{MLE}}, \quad (6.13)$$

which, using the variance property of Poisson random variables, should reach a value close to unity when the MLE estimate is within the statistical similarity expected. In practice, for the results of section 3 the algorithm was stopped when this χ^2 reduced by less than 1% from one iteration to the next. Thus usually resulted in convergence in around 40 iterations (see figure 6.4)), although the image showed little change if a further 40 iterations were performed indicating that the algorithm is reasonably stable.

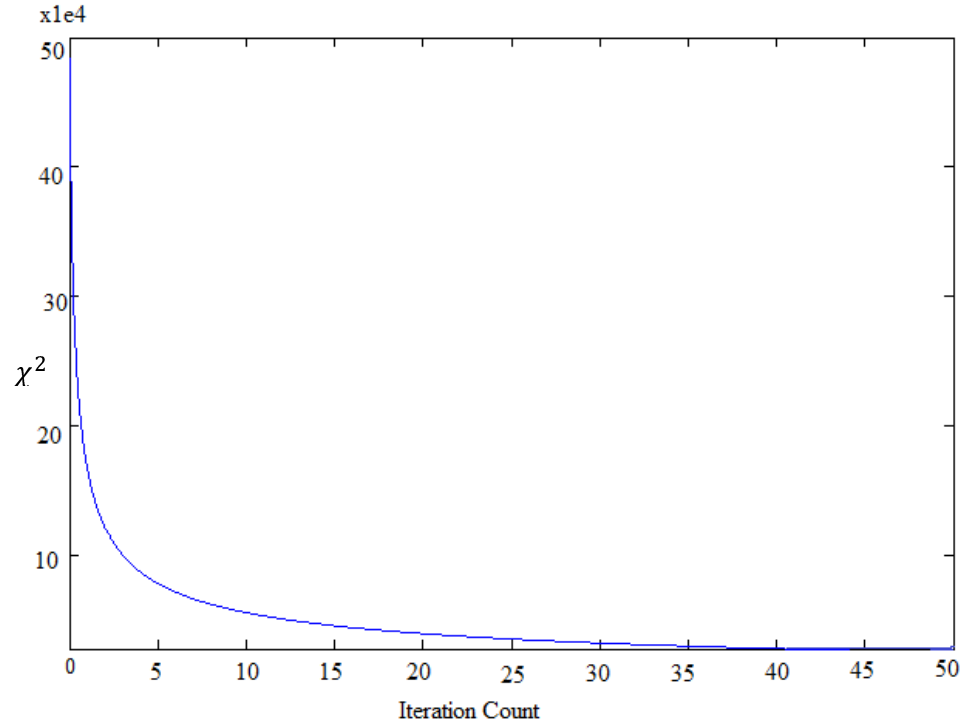


Figure 6.4: χ^2 statistic showing that the ML algorithm convergence is achieved in approximately 40 iterations.

6.5 Simulation results

The aim of this section is to compare the output of the novel data collection and processing methods outlined in sections 6.1 and 6.3 with the images that would have been constructed with a conventional pinhole detection method. As mentioned in section 6.1, MLE methods have already been applied to conventional CFM images, so MLE restoration on the CFM (pinhole) image data is also performed for comparison. A simple 3D object consisting of a planar circle and a planar cross separated in the z direction by $4\text{ }\mu\text{m}$ is used as the test object and is shown in figure 6.5.

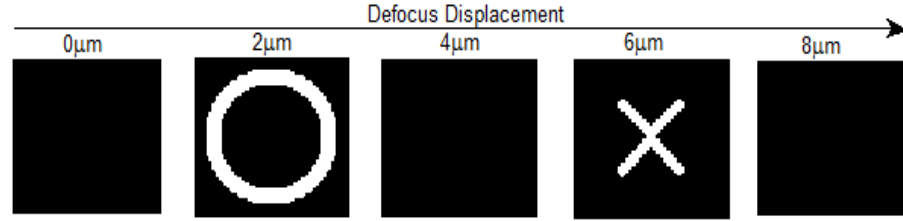


Figure 6.5: Simulated 3D object with an annular ring structure at $z = 2 \mu\text{m}$ and cross shaped structure at $z = 6 \mu\text{m}$

Data was computer generated by applying Fourier optics imaging equations [5, 43] to this object and then by use of a Poisson random number generator to simulate the detection process. The object fluorescence was normalised, initially, so that the sum of all the recorded data sets has an expectation value of 2,000 photon events. These data were then used in two ways. Firstly the photon counts within a certain radius were added together (and those without that radius neglected) to simulate a pinhole detection method and to directly form images; secondly the identical data (all photon counts) were processed by the method described in the previous section. A flowchart of the method is presented in figure 6.6 for quick reference.

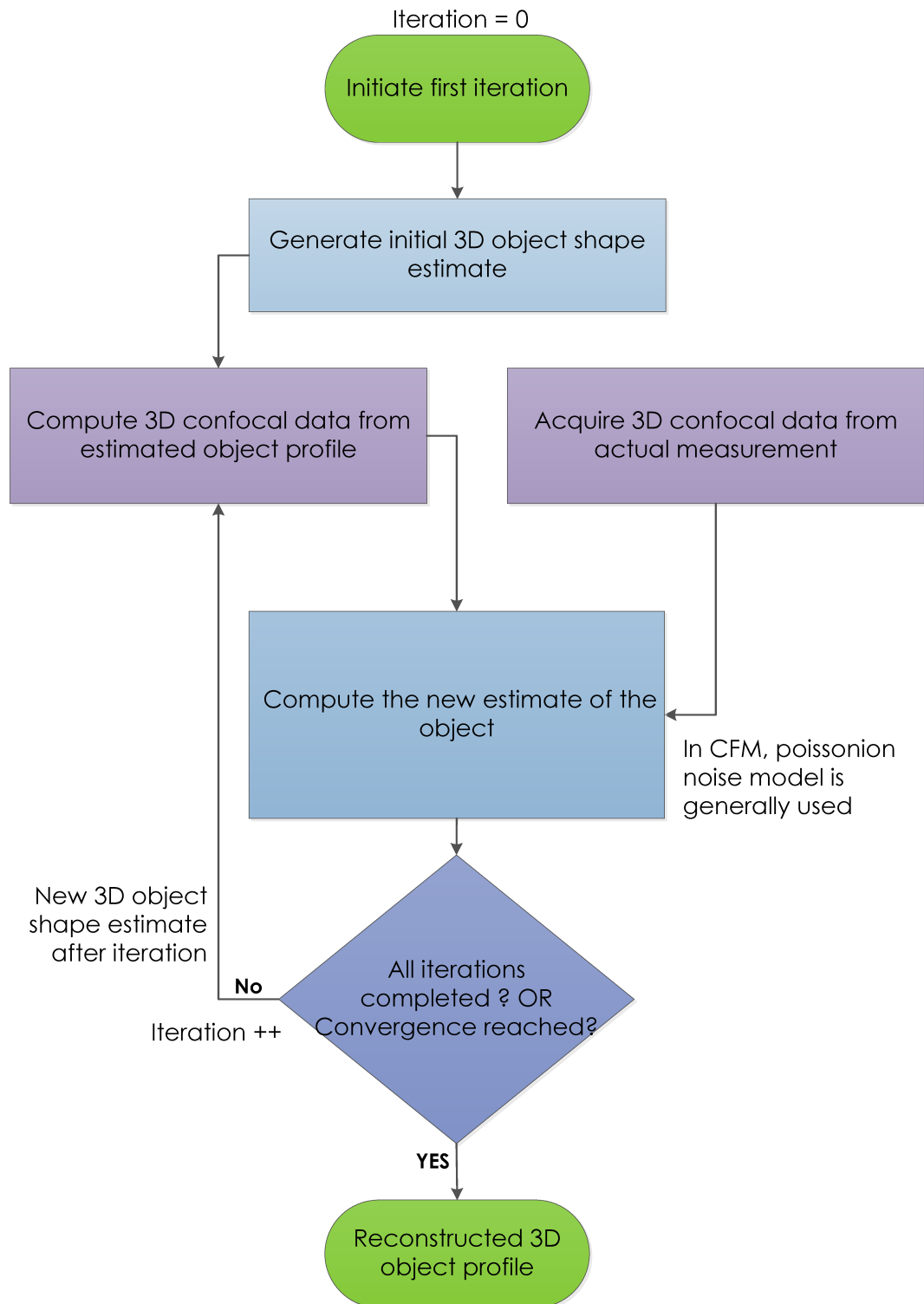


Figure 6.6: Flowchart showing how the ML algorithm iteratively estimates the 3D object profile

A conventional CFM yields the best resolution when the pinhole radius is made small. Figure 6.7 demonstrates how increasing the pinhole radius in a conventional CFM (with $NA = 0.5$ and $\lambda = 0.5 \mu m$) has two effects; firstly the images can be seen to be less noisy as more photons are allowed through the pinhole, but secondly it can be seen that the resolution is compromised as the pinhole becomes less effective in excluding non-confocal light. In this case a relatively small number of photon emissions is simulated.

Using the noisy images shown in figure 6.7 as data, a maximum likelihood restoration algorithm was applied to these images. This method of restoration has been previously applied to various microscopy techniques [1-23] the analytic expressions for which are similar to those derived in section 6.3. The results are shown in figure 6.8 and it may be seen that an improvement in both lateral and axial resolution is demonstrated together with an improvement in the signal-to-noise ratio of the processed images.

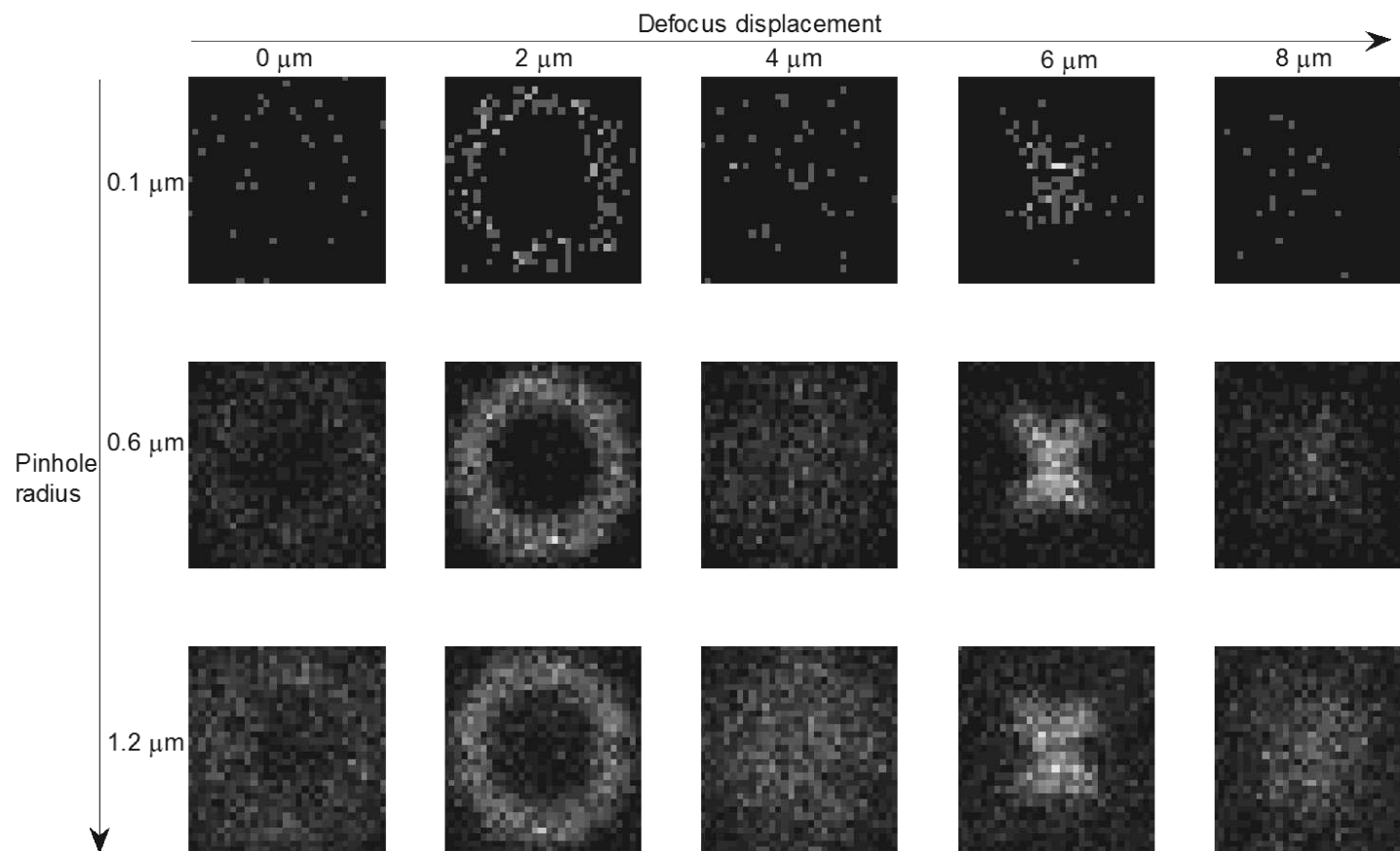


Figure 6.7: Simulated conventional (pinhole) CFM images of object shown in fig 5.3 at different pinhole radii. Here again $\text{NA} = 0.5$ and $\lambda = 0.5 \mu\text{m}$ and the expected number of detected photon events in the complete data set is 2,000.

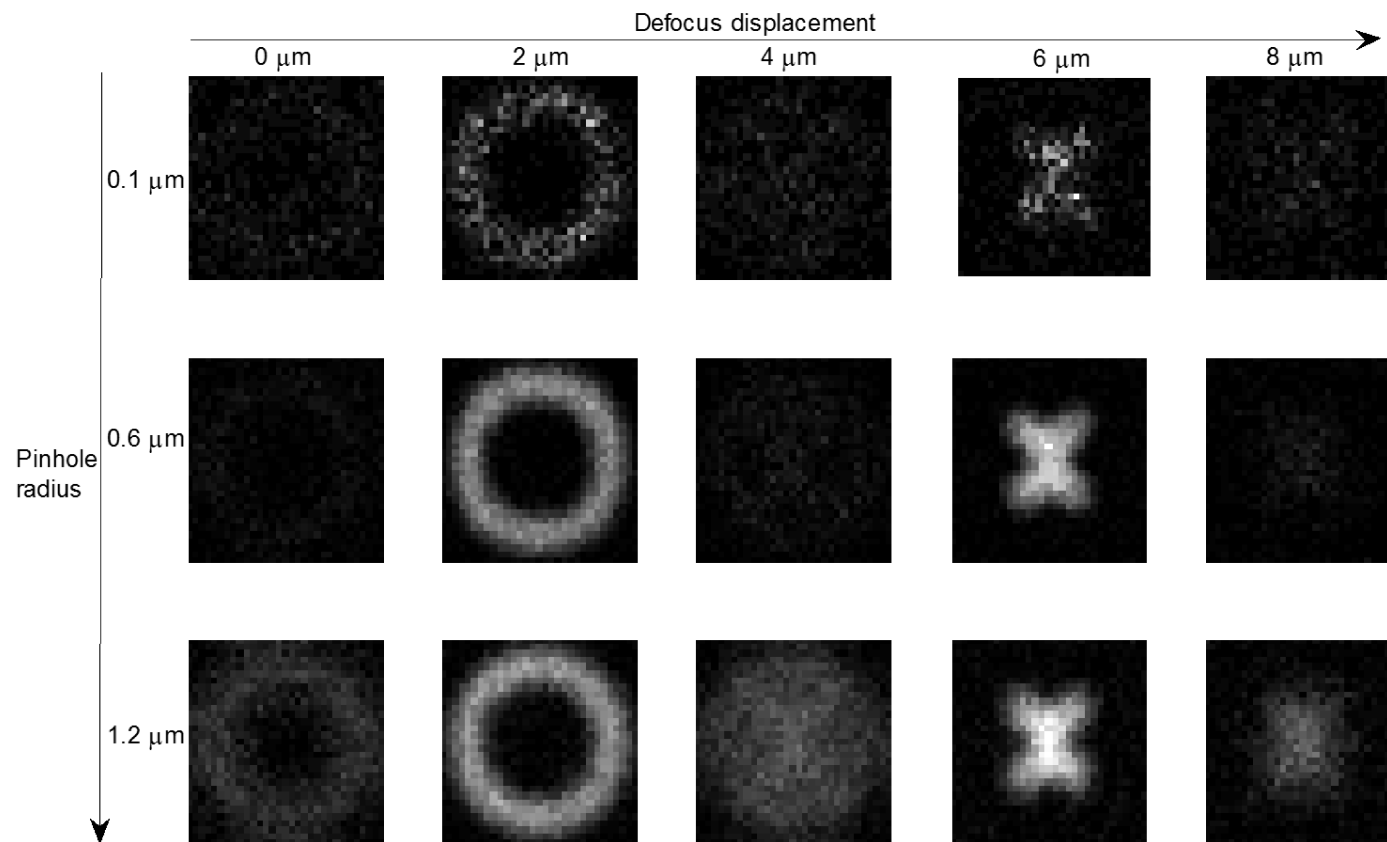


Figure 6.8: Reconstructed CFM images using maximum likelihood processing applied to data shown in fig 6.7 ($\text{NA} = 0.5$ and $\lambda = 0.5 \mu\text{m}$). Clearly the MLE processing enhances resolution.

Now, using all the photon data (recorded in a 64x64 detector array) in place of the detection pinhole, the algorithm of equation (6.12) is applied. The results are shown in figure 6.9; and they show a significant improvement compared to those presented in figures 6.7 and 6.8 both in terms of resolution (radial and axial) and in terms of image signal-to-noise ratio.

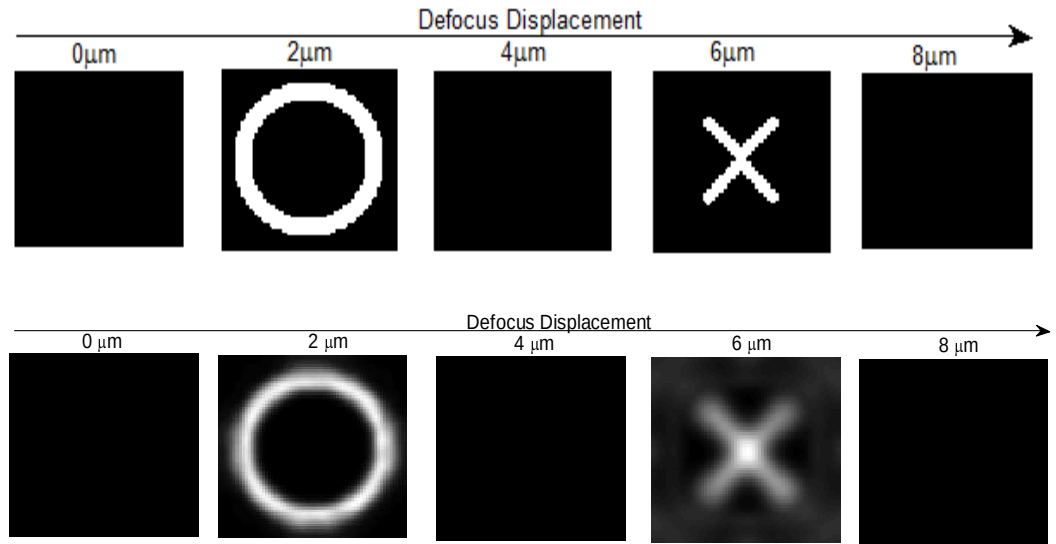


Figure 6.9: Top row – Simulated 3D object (reproduced from figure 6.5 for comparison). Bottom row - reconstructed CFM images using maximum likelihood processing applied to data recorded using an array detector (i.e. the same data as processed to form figures 6.7 and 6.8). By comparing these images with fig 6.7 and fig 6.8 the 3D resolution improvement achieved here should be apparent.

The improvement over the images in figure 6.8 could be due to, firstly, the use of the MLE restoration and, secondly, the fact that additional photon counts are being used in the data set. The fact that the images in figure 6.9 are an improvement over those in figure 6.8 suggests that at least part of the improvement comes from the successful employment of the data contained in those additional photon counts.

To demonstrate the effectiveness of the technique at different photon levels, the simulations and processing were repeated but using an increased photon count value where the object fluorescence was normalised, so that the sum of all the recorded data sets has an expectation value of 20,000 photon events; the results are presented in figures 6.10 to 6.12. It may be seen that, again, the results of processing the array detector data has produced an image with increased resolution and signal-to-noise ratio compared both with the images from conventional (pinhole) confocal; raw and MLE processed.

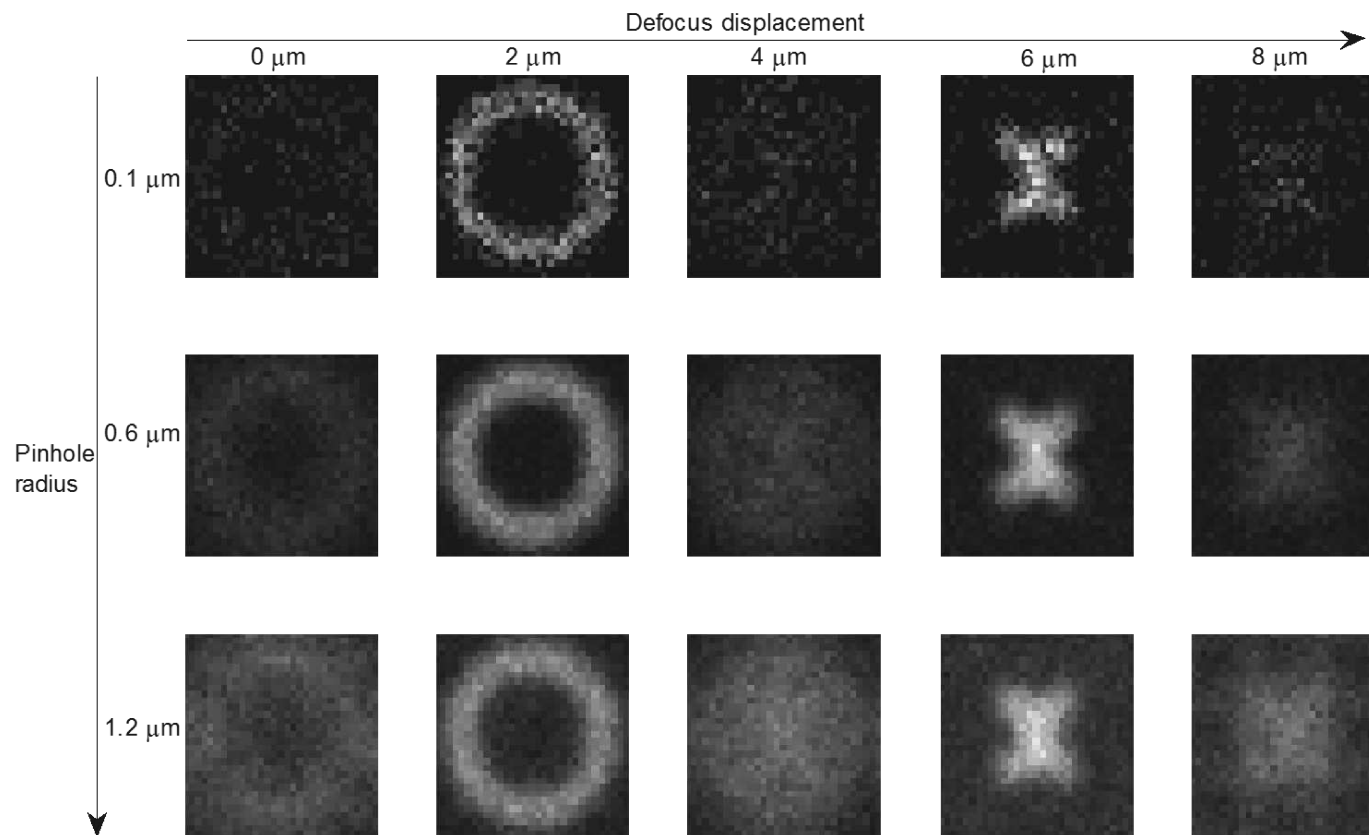


Figure 6.10: Simulated CFM images of object shown in fig 6.5 at different pinhole radii. Here again $\text{NA} = 0.5$ and $\lambda = 0.5 \mu\text{m}$ and the expected number of detected photon events in the complete data set is 20,000.

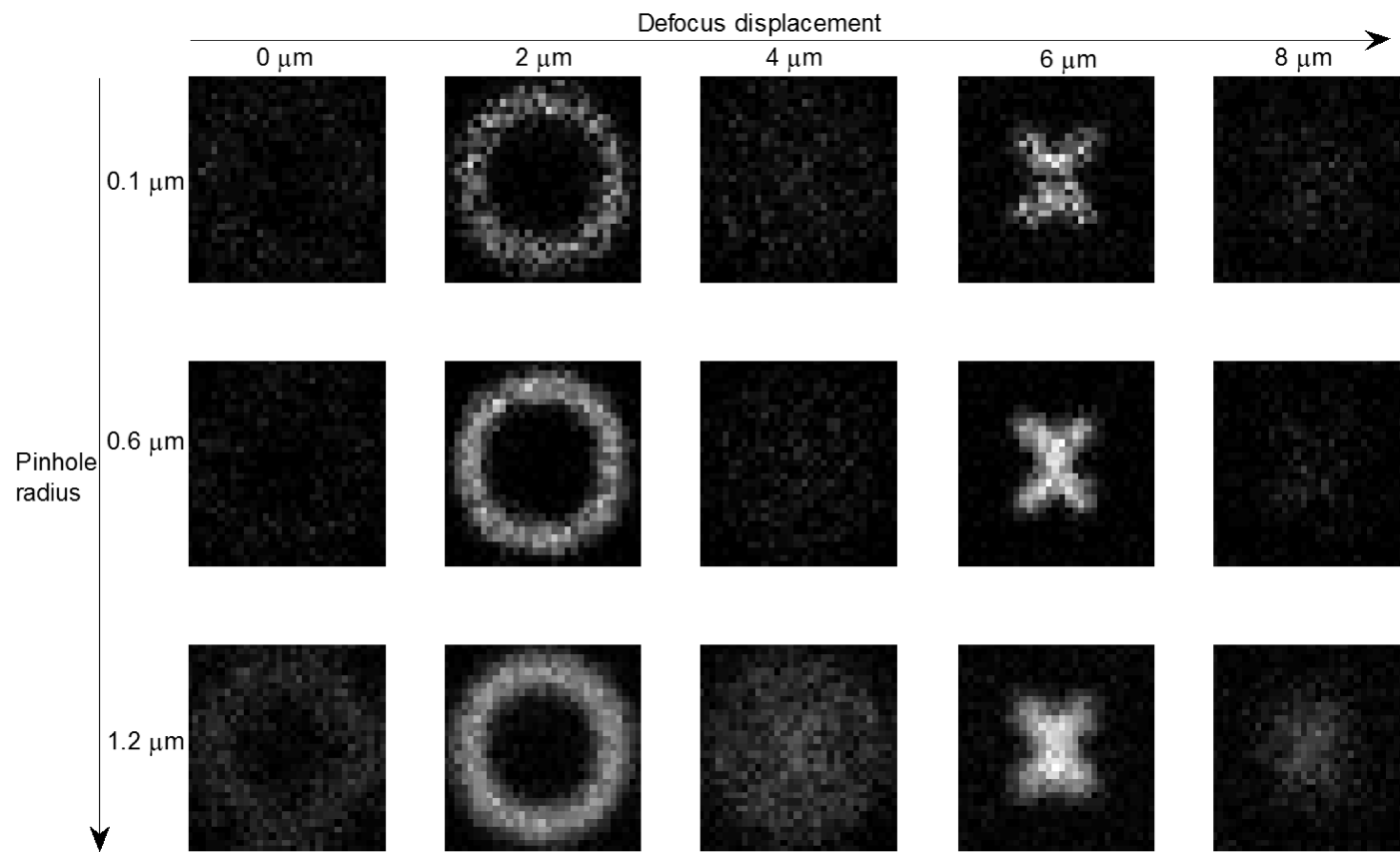


Figure 6.11: Reconstructed CFM images using maximum likelihood processing applied to data shown in fig 6.10 ($\text{NA} = 0.5$ and $\lambda = 0.5\mu\text{m}$).

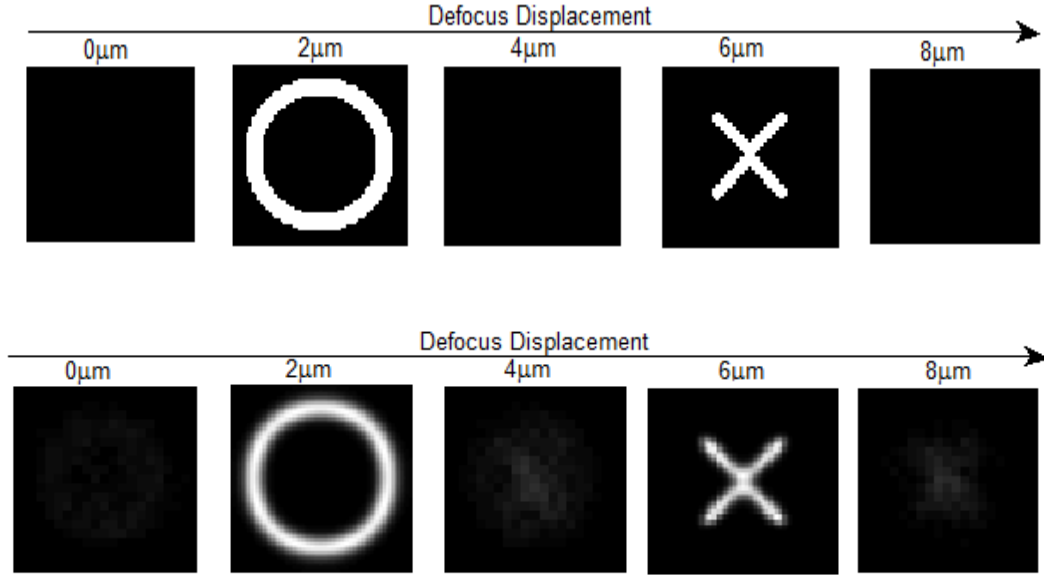


Figure 6.12: Top row – Simulated 3D object (reproduced from figure 6.5 for comparison) Bottom row- reconstructed CFM images using maximum likelihood processing applied to data recorded using an array detector (i.e. the same data as processed to form figures 6.10 and 6.11). By comparing these images with fig 6.10 and fig 6.11 the 3D resolution improvement achieved here should be apparent.

6.6 Discussion and conclusions

Use of an array detector combined with maximum likelihood processing has been shown to simultaneously improve both noise performance as well 3D resolution of a confocal fluorescence microscope. The improvement has been shown by comparing simulated images generated using the proposed method with those acquired using conventional (pinhole based) CFM systems as well those employing ML based reconstruction of conventional CFM images.

Even under low object fluorescence (which leads to fewer photon detection events) the proposed method has demonstrated its ability to offer images with enhanced resolution and signal-to-noise ratio performance. This has obvious benefits for live

cell imaging wherein the samples are vulnerable to photodamage. Figure 6.13 shows two more examples of the proposed method when the total number of recorded photons is limited to 2000.

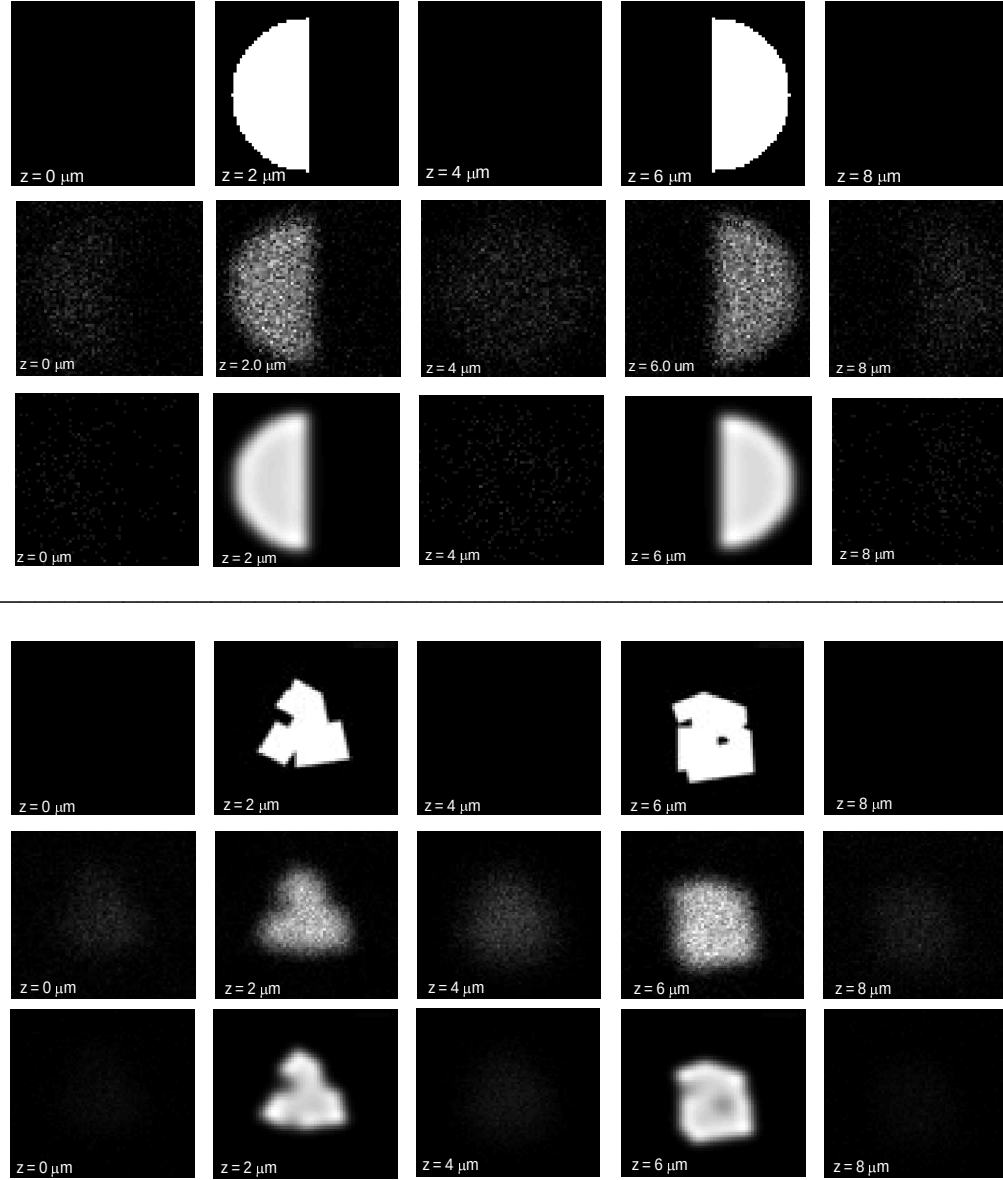


Figure 6.13: Top and bottom panel show CFM images of two different simulated 3D objects; In each panel: Top row – Simulated 3D object consisting of two planar structures separated by $4 \mu\text{m}$. Middle row – CFM images of the simulated object with a very small pinhole radius of $0.1 \mu\text{m}$. Bottom row – Reconstructed images of the simulated object using ML processing and an array detector. ML processing has been applied to the same data that was used to produce conventional CFM images. Total number of recorded photon events in the imaging of the simulated objects is 2,000 ($\text{NA} = 0.5$ and $\lambda = 0.5 \mu\text{m}$).

Like other image reconstruction algorithms, the proposed method requires significant post-detection processing demanding relatively long computer run times. Using a desktop PC the simulation results presented in figures 6.9 and 6.12 took around two hours to produce. However, owing to the modular nature of the ML algorithm, run time has the potential to be considerably reduced as demonstrated by Conchello [36].

Also the large volume of detector data that must be recorded at each scan position adds considerable data-collection time; for a large pixel-number image this could lead to lengthy data collection times as most array detectors are slower to read than a single element detector used in conventional CFM. A possible way to mitigate this problem, may be to use some form of structured illumination pattern instead of the simple illumination simulated above, for example if an array of focussed beams were employed then the number of scan positions and images recorded could be very significantly reduced. The next section will investigate the effect this might have on the resulting image quality and computation time.

6.7 Using structured light for faster ML processing

Using a structured illumination pattern, such as that formed using an array of equally spaced pinholes; the data collection time can be substantially reduced. Consider the pattern shown in figure 6.14 (b) which consists of an array of small pinholes separated by $16\text{ }\mu\text{m}$ (here $\text{NA} = 0.5$ and $\lambda = 0.5\text{ }\mu\text{m}$). By moving the laser in a raster scan fashion (as indicated by the red arrows in figure 6.14 (b)), a much smaller volume of data is recorded and this considerably speeds up the ML

processing time. Figure 6.14 (c) shows the resulting structured light pattern formed by convolving the illumination PSF (figure 6.14 (a)) with the pattern (figure 6(b)).

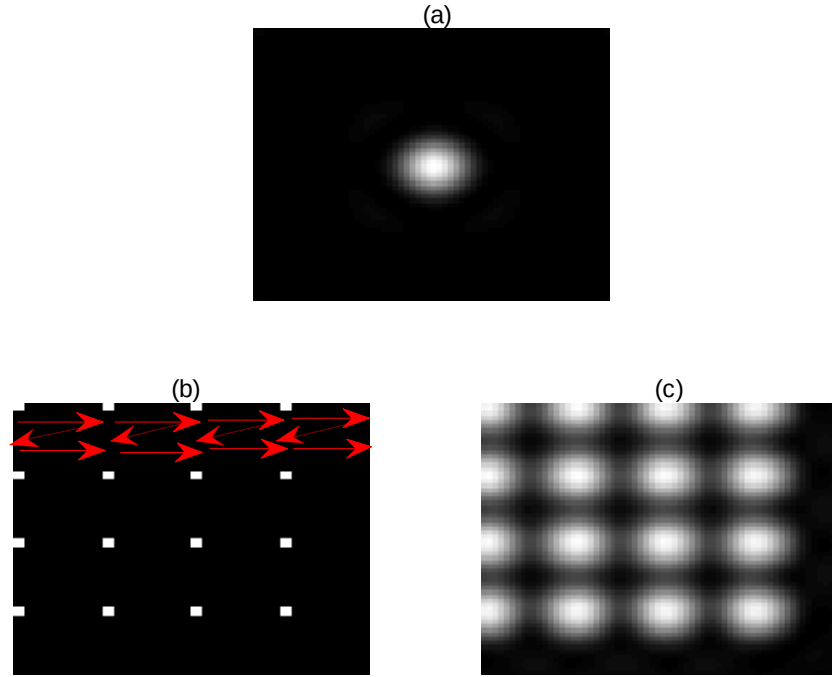


Fig 6.14: (a) Illumination lens PSF (b) Pattern of point sources used to generate the illumination (c) Structured illumination pattern as a result of using (b). Here $NA = 0.5$ and $\lambda = 0.5 \mu m$.

For a 64x64 detector array, figure 6.15 shows that this method suffers from almost no loss in image quality for the same simulated object used in figure 6.13 (bottom panel). The simulation run time for 40 iterations was found to be approximately 15 mins which is about 4 times faster than the point scanning approach described earlier. Thus, by using structured light, substantial speed improvements can be made.

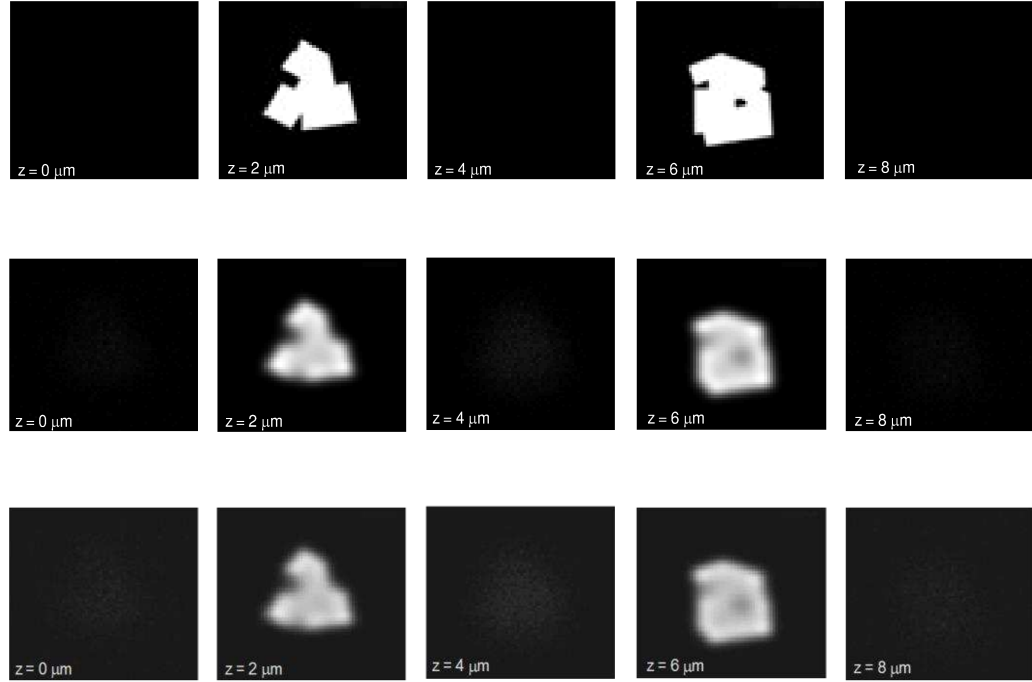


Figure 6.15: Top row – Simulated 3D object (reproduced from figure 6.13 (bottom panel) for comparison) Middle row- reconstructed CFM images using maximum likelihood processing of the object applied to data recorded using an array detector and point scanning (reproduced from figure 6.13 (bottom panel) for comparison). Run time clocked as about 2 hours. Bottom row – reconstructed CFM images using ML processing using structured light pattern shown in figure 6.14. Run time clocked as about 15mins.

It would be interesting to explore different structured light patterns to identify those that may offer an improvement in image quality while also providing the speed improvement. This will be considered for future work and highlighted in the next chapter.

6.8 Chapter Summary

- For its intrinsic non-negativity constraint, simple regularisation, ability to restore high-noise images and numerical stability the ML algorithm has been

previously evidenced to show image enhancement in several fluorescence microscopy techniques.

- Processing images of an array detector based CFM system using Maximum Likelihood processing is capable of offering enhanced resolution and signal-to-noise performance. Analytical derivation of the proposed method has been presented.
- The performance improvement has been shown to be considerably better than that achieved using ML processing applied to conventional pinhole based CFM systems. A paper [44] has been submitted to IOP Measurement Science and Technology and is currently under review.
- The proposed method is well suited for live-cell imaging where photodamage can be a major concern because of its demonstrated ability to produce good quality images under low light.
- Long processing times that are inherent to image reconstruction techniques can be reduced through implementation of faster algorithms (such as demonstrated by Conchello) which make use of the modular nature of the ML algorithm.
- By using some form of structured illumination pattern, such as an array of focussed beams, the number of scan positions and images recorded can be significantly reduced which reduces the processing time.

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Chapter 7

Conclusions and Future Work

Optical microscopy allows the non-invasive study of biological specimens with the naked eye [1]. For this primary reason optical microscopy is often preferred over electron microscopy (EM) which although it offers higher resolution than optical microscopy, subjects the sample to high energy beams and invasive treatment prior to imaging. Conventional widefield fluorescence microscope systems are diffraction limited to a resolution of ~ 200 nm which greatly limits their applicability to bio-imaging [2]. Several advanced techniques such as STORM [3, 4], PALM [5, 6], SIM [7] and STED [8] have been developed over the past decades but these are expensive, complex and often demand high sample exposure times making them ill-suited for live cell imaging applications. Fluorescence microscopy has proven to be a powerful and versatile imaging tool for biologists with its ability to tag virtually every molecular protein. The fluorophore molecules, being very bright, are easily distinguishable from other background signals, thus facilitating high-contrast imaging.

This thesis focussed on one of the most fundamental yet significant optical microscopy techniques – confocal fluorescence microscopy (CFM). CFM, a robust and less complex alternative to the above mentioned microscopy technique, has proven to be an indispensable tool for bio-imaging for its superior optical sectioning capability (ability to produce clear images of focal planes deep within a thick sample). A CFM system was studied in this thesis by analysing its performance with respect to the inherent trade-off

between the two most important metrics - Signal-to-noise ratio and 3D optical resolution. Using alternative detection schemes, new pinhole geometries and image reconstruction methods, the thesis presented, through computer simulation, results demonstrating how performance improvements can be realised in a CFM system. This chapter will draw conclusions from the thesis and identify future work.

7.1 Conclusions

Following an outline of the thesis and listing of the key contributions to knowledge in chapter 1, chapter 2 presented a detailed background review of optical microscopy explaining its most important principles and terminologies. The many high resolution microscopy techniques were also reviewed, highlighting their advantages and limitations. Chapter 3 explained the fundamental setup, the image formation process (with relevant analytical expressions) and design considerations pertaining to a conventional CFM system. The impact of the detection pinhole geometry on the performance of a CFM was examined by means of a set of effective trade-off curves – one for the lateral resolution vs SNR trade-off and another for the axial resolution vs SNR trade-off.

The resolution was calculated by measuring the FWHM of the system's response to a point or planar object and the SNR calculated under shot noise limited detection (shot noise is well known to be the chief limiting noise contributor in CFM systems [9]). By means of these trade-off curves the chapter identified “regions of interest” (ROIs) along the trade-off curves, operating in which would indicate an improved system performance i.e. better

SNR for a given resolution, better resolution for a given SNR or better SNR as well as resolution.

In Chapter 4, using alternative pinhole geometries in conjunction with new detection schemes, it was shown how performance gains can be realised in both the lateral and axial directions. ROI operation (in both the lateral and axial directions) was demonstrated using two families of pinholes – triangular and generalised normal (GN) shapes. Using MATLAB's (version 2014 and higher) multi-objective genetic algorithm function `gamultiobj` (implementing a form of the non-dominated sorting genetic algorithm (NSGA) called NSGA II [10, 11]), a rigorous computational search of all possible pinhole geometries was performed to find a set of Pareto optimal pinhole shapes. The Pareto shapes identified were found to be well matched to the triangular as well as the GN pinhole family. The chapter thus revealed the potential of using alternative pinhole geometries for realising performance gains in CFM systems.

Chapter 5 examined a recently developed detection scheme called subtractive imaging; wherein a special annular pinhole was used to divide the confocal point spread function signal between two detectors. By using fast point detectors in place of CCD arrays, it was shown how using numerical optimisation yields an optimum “differential pinhole” (ODP) to achieve considerable 3D resolution gains over conventional (circular pinhole based) CFM systems. A new resolution metric termed as equivalent radius (ER) was introduced to overcome the limitation of the FWHM measure to account for the unwanted negative sidelobe intensities that are encountered in this approach.

By examining the trade-off curves it was also shown that the proposed design is able to offer simultaneous and maximum performance gains up to a considerably high SNR in comparison to conventional (circular pinhole) based CFM systems (i.e. operation in the ROI). Table 7.1 compares the resolution achieved by using pinhole geometries, each at an SNR of 0.5, proposed in chapters 4 and 5 with the resolution of a conventional (circular) pinhole based CFM system.

Table 7.1: Comparison of the 3D resolution (at an SNR of 0.5, NA = 0.5 and $\lambda = 0.5 \mu\text{m}$) achieved in a CFM system using different detection schemes.

Pinhole type	Detection type	Lateral resolution (μm)	Axial resolution (μm)
Conventional (circular) pinhole	Single point detector	0.4	2.9
Triangular pinhole (defined by equation 4.10 with $n_e = 1$)	Array detector	0.37	2.87
Generalised normal pinhole (defined by equation 4.11 with $n_e = 4$)	Array detector	0.38	2.83
Optimised differential pinhole (defined by equation 5.1 with $\gamma = 1$ and $\frac{r_p}{r_t} = 1.41$)	Two point detectors	0.34	2.0

It can be seen that between the different detection schemes developed in chapters 3, 4 and 5, the ODP geometry provides the maximum performance improvement, yielding a lateral and axial resolution gain of 17% and 45% respectively. It is interesting to note that, using array detection, although both the triangular and GN pinhole geometries offer 3D resolution improvements, one geometry offers better lateral than axial resolution and vice versa. As demonstrated in chapter 3, by performing a genetic algorithm based search of all possible pinhole geometries, it is possible to find other pinhole geometries that can offer higher resolution (than triangular/GN based pinhole geometries) along either lateral or axial direction.

Even under high background noise, the optimised differential pinhole system continued to demonstrate performance gains in either direction. By requiring no significant alteration to the microscope architecture, pupil filters offer a relatively easy and low cost alternative (to the more complex super-resolution techniques such as 4Pi and STED for example) to sharpen the 3D PSF. Coupled with a recently developed alternative pupil filter geometry – a fan shaped pupil set, further performance enhancements along the axial direction were demonstrated using the ODP based CFM system.

Lastly, in Chapter 6 the thesis proposes the use of a deconvolution technique and an alternative detection scheme to demonstrate substantially higher improvements in the quality of images acquired by a CFM system. An image reconstruction technique, based on an expectation maximisation maximum likelihood (EM-ML) algorithm was used in conjunction with array detectors to demonstrate enhanced resolution and noise performance of a CFM system. The point scan method used here renders the algorithm slow with long

run times. To mitigate this, structured illumination was used to show how similar resolution gains in the array detector based CFM systems could be realised but in a much shorter time. For a 64x64 detector array, using structured illumination reduced the run time from a couple of hours to approximately 15 mins, a substantial speed improvement.

7.2 Future work

In this section, some suggestions for further work in the areas explored in this thesis are presented.

7.2.1 Maximum-likelihood based image reconstruction of the optimised differential pinhole based CFM images

- The performance gains achieved using the optimised differential pinhole based CFM system over conventional circular pinhole point detector based CFM systems has been demonstrated in chapter 5.
- Further, chapter 6 demonstrated how using image reconstruction based on Maximum likelihood processing performance gains be achieved.
- By amalgamating the above two techniques, even further resolution improvements can be anticipated and this would be the subject of immediate work.
- Furthermore an investigation into the use of different (possibly more complex than the one simulated in chapter 6) structured illumination patterns would help identify those that offer the best trade-off between optimisation run time and image quality.

7.2.2 Characterising performance of other microscopy systems

- By modelling the imaging process of other microscopy techniques it is possible to characterise their performance using similar trade-off curves as presented in chapter 3.
- It would be interesting to investigate the potential of using non-circular pinhole geometries and alternative pupil filters to seek performance gains in these other microscopy techniques.

7.2.3 Developing a portable image optimisation software package

- It would be interesting to create a portable image optimisation tool – a software package with a library of mathematical models of different microscopy systems that allows the end user to automatically optimise a given image output from their microscope of choice.
- More accurate microscope imaging models than the ones developed in this thesis would be developed by using vectorial theory (suitable for high NA systems) for light scattering in thick samples, by accounting for lens aberrations effects and other optical misalignment errors.

7.2.4 Developing performance metrics based on Fisher Information theory

Another approach to establishing the performance limit of a microscope has been developed by Ram [12] who used the Fisher information theory to provide a lower bound on the precision with which an unknown parameter θ can be evaluated. This bound is concerned with the Cramer-Rao inequality [13] that states the variance of any unbiased estimator θ is always greater than or equal to the associated Fisher information matrix.

$$\text{Var}(\theta) \geq I^{-1}(\theta). \quad (7.1)$$

Thus the performance limit with respect to a particular object's attribute (e.g., photon detection rate, object location, level of defocus etc.) is determined by the standard deviation of the inverse Fisher information matrix i.e. by taking square root of 7.1. If the attribute is the position then this gives a limit to the accuracy with which the object's position can be found [12, 14].

In optical microscopy the Fisher information matrix over a fixed acquisition period $[t_0, t]$ is given by [14],

$$I(\theta) = \int_{t_0}^t \int_C \frac{1}{A_\theta(\tau) f_{\theta,\tau}(r)} \left(\frac{\partial [A_\theta(\tau) f_{\theta,\tau}(r)]}{\partial \theta} \right)^T \frac{\partial [A_\theta(\tau) f_{\theta,\tau}(r)]}{\partial \theta} dr d\tau. \quad (7.2)$$

where $A_\theta(\tau)$ is the photon detection rate of the Poisson process modelling the time points of photon arrival at detector C. $f_{\theta,\tau}(r)$ represents the PDF (probability distribution function) of independent random variable mapping to the spatial position of the detected photons. It is assumed here that the temporal and spatial co-ordinates are mutually independent. Being a function of time $f_{\theta,\tau}$ is applicable to moving objects and thus $I(\theta)$ represents a general expression for the Fisher information. Ram [14] also presents a time invariant expression for static objects. For two- point resolution $I(\theta)$ is given by,

$$\begin{aligned} I(d) = \frac{1}{4} \int_{t_0}^t \int_{R^2} \frac{1}{A_1(\tau) q\left(x + \frac{d}{2}, y\right) + A_2(\tau) q\left(x - \frac{d}{2}, y\right)} \times \\ \times \left(A_1(\tau) \frac{\partial q\left(x + \frac{d}{2}, y\right)}{\partial x} \right. \\ \left. - A_2(\tau) \frac{\partial q\left(x - \frac{d}{2}, y\right)}{\partial x} \right)^2 dx dy. \end{aligned} \quad (7.3)$$

Here d is the separation between two point objects and $q(x,y)$ is an image function given by 7.4. $J_1(x,y)$ is the first order Bessel Function of the first kind.

$$q(x,y) = J_1^2\left(\frac{\frac{2\pi \cdot NA}{\lambda} \sqrt{x^2 + y^2}}{\pi(x^2 + y^2)}\right), \quad (7.4)$$

where λ is the wavelength of light and NA is the numerical aperture of the objective. Using 7.4 in 7.3 with both point sources having equal photon detection rates A_d the resolution limit σ_d is given by taking the inverse of the square root of the Fisher Information. Thus,

$$\sigma_d = \frac{1}{\sqrt{4\pi \cdot A_d \cdot (t - t_0) \cdot B_d(d)}} \frac{\lambda}{NA}, \quad (7.5)$$

with $B_d(d)$ given by,

$$\begin{aligned} B_d(d) = \int_{R^2} & \frac{1}{\frac{J_1^2(\alpha, r_{01})}{r_{01}^2} + \frac{J_1^2(\alpha, r_{02})}{r_{02}^2}} \\ & \times \left(\left(x + \frac{d}{2} \right) \cdot \frac{J_1(\alpha, r_{01}) \cdot J_1(\alpha, r_{02})}{r_{01}^2} \right. \\ & \left. - \left(x - \frac{d}{2} \right) \cdot \frac{J_1(\alpha, r_{01}) \cdot J_1(\alpha, r_{02})}{r_{02}^2} \right)^2 dx dy \end{aligned} \quad (7.6)$$

Where the wave number $\alpha = \frac{2\pi \cdot NA}{\lambda}$ and $r_{01}^2 = \left(x + \frac{d}{2}\right)^2 + y^2$ & $r_{02}^2 = \left(x - \frac{d}{2}\right)^2 + y^2$. Equation 7.5 thus gives the fundamental resolution limit of the localisation accuracy of the object under an idealised situation i.e. a microscope system without adding noise sources. A performance metric thus based on Fischer information theory could be used to evaluate and compare the performance of different optical microscopy systems.

7.2.5 Experimental verification

Lastly, experimental verification of the performance gains offered by the optimised differential pinhole based CFM system as well as that offered by the use of non-circular pinhole geometries is of course worthy future work. It would also be interesting to experimentally investigate the potential of using a differential pinhole with other microscope systems such as SIM and 4Pi microscopy.

7.3 References

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