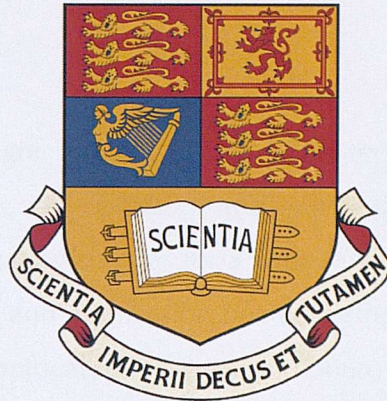


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Microscopic Imaging and Photo-stimulation using Micro-structured Light Emitting Diodes

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Thesis submitted in partial fulfilment of the requirements for the degree of Doctor of
Philosophy of the University of London

July 14, 2008

Abstract

Recent developments in light emitting diode (LED) technology such as increasing improvements in brightness, UV operation and micro-structured array devices make LEDs very attractive illumination sources for a wide range of applications. This thesis describes the development and application of micro-structured LED technology as a patterned light source for confocal microscopy and photo-stimulation of neuron cells.

Using lithographic techniques, light emitting diodes can be readily patterned into arrays of individual elements of order 20 microns in size. Several such programmable arrays are presented and tested, along with the ancillary drive electronics that were developed to allow the display of arbitrary light patterns on a timescale of tens of microseconds.

A one-dimensional 120-element array of line emitters, is applied to obtain optically sectioned images in a fluorescence microscope with no moving parts. Several optical sectioning techniques are demonstrated, including grid-projection structured illumination microscopy and line scanning confocal microscopy. This latter technique is extended to multi-line illumination, and the concern about removing the remaining out-of-focus background leads to a new background subtraction technique that dramatically improves the sectioning strength and image contrast. The theory of optical sectioning by out-of-focus background subtraction under line-patterned illumination is developed, and it is shown that the performance converges towards that of the grid-projection structured illumination method for high fill-factor illumination patterns. This theory is used to propose the design of a double-sided spinning disk microscope that should enable confocal imaging at very high-frame rate with superior optical sectioning.

A second application makes use of matrix addressable LED arrays for direct stimulation of neuron cells. Gallium-Nitride LED arrays are demonstrated to be realistic light sources for photo-stimulation that have clear advantages in terms of emission wavelengths, temporal response and spatial resolution. Micro-LED arrays enable very reliable photo-stimulation with sub-cellular resolution at a single cell level and selective neuron excitation at a network level. A long-term project to implement an LED based retinal prosthesis is presented.

To my parents

To my family

To Constance

"Without question, the greatest invention in the history of mankind is beer. Oh, I grant you that the wheel was also a fine invention, but the wheel does not go nearly as well with pizza."

Dave Barry, Pulitzer Prize winner, 1988

Acknowledgements

First I would like to express my gratitude to my supervisors Dr. Mark Neil and Professor Paul French for giving me the opportunity to work with them and learn from them in this exciting field that is photonics, at the interface between modern physics, instrumentation, biology and neuro-science. No better team could have supervised this work, always simple, available and very British. I genuinely enjoyed our professional interactions, personal discussions and generous group dinners. These acknowledgements do not symbolize a farewell but surely the beginning of a new story.

Thanks to Erdan Gu and Martin Dawson for the constructive interactions and successful collaboration we had. I sincerely enjoyed working with Khadija, Valerie, Nahed, Dan, Chris, Ian, Peter, Pieter, James, Clifford, Ewan, Richard, Dylan, Stephane, Sunil, David, Hugh, Tom, Konstantin and Patrick, and travelling with them both abroad for conferences, or a bit closer to the Holland Club. Thanks to Egidijus and Bosanta for their company and friendship during all this time, and for so many memories, with a kilt or otherwise. Thanks to Nir, Gordon, Matt and Juan for the exciting work we did together. A special thank you must go to Gordon and Valerie in their kind efforts for proofreading this work.

The EPSRC must have a mention for funding my PhD studentship and offering me opportunity to conduct my research work with both serenity and freedom. Thanks to all the members of the optics workshop, in particular Martin and Simon, who also allowed this work to be carried out in the best possible conditions.

Thanks to my London friends for the great time we had, and especially to the male members of the Holyport Road brotherhood, Cedric and Vincent. It is not only memories we share but also a most significant period of our lives.

And last but not least, thank you to Constance, whose love and patience supported this work. I realise every day how lucky I am.

Acronyms

OTF	Optical transfer function
PSF	Point spread function
PSCM	Point scanning confocal microscope
LSCM	Line scanning confocal microscope
SIM	Structured illumination microscopy
SPIM	Single plane illumination microscopy
DSI	Dynamic speckle illumination microscopy
LED	Light emitting diode
NA	Numerical aperture
UV	Ultra-violet
FWHM	Full width at half maximum
PWM	Pulse width modulation
SPI	Serial port interface
SNR	Signal to noise ratio
CCD	Charge coupled device
CMOS	Complementary metal–oxide–semiconductor
ChR2	ChannelRhodopsin 2

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Chapter 1 Introduction and thesis overview

1. Introduction

Confocal microscopy was invented almost fifty years ago [1], however, since then it has largely remained an imaging technique used by microscopy specialists only. By scanning a focused laser beam onto object and passing the reflected or fluorescence light through a confocal detection pinhole, confocal microscopy allows images with superior lateral and axial resolutions to be reconstructed at any depth inside the sample. Blocking the out-of-focus light enables true optical sectioning and thus subsequent three-dimensional reconstruction of micrometer size objects [2, 3]. The advances in laser light sources, electronics and photo-detectors has more recently enabled commercialisation of confocal systems and these have been used to unravel many fundamental issues in biology, semi-conductor physics, and other related fields where imaging fine object structures is crucial. When used together with fluorescent makers, confocal microscopes make it possible to track particular molecules of interest and optically image very fine internal cellular structures with unprecedented resolution.

Commercially available, point scanning confocal microscopes (PSCM) suffer from several inherent drawbacks that make them rather expensive and challenging to use: laser sources are still expensive and often many lasers operating at different wavelengths are integrated in the confocal system to excite different fluorophores; highly accurate scanning systems and efficient photo-detectors are critical to obtain a decent image quality and can also be very expensive; the requirement to scan the specimen/beam one pixel at a time across the whole field of view introduces severe limitations to the acquisition speed. In an attempt to overcome these limitations, several techniques have emerged that enable faster, cheaper and less complex imaging.

The first category of confocal-like imaging systems tried to increase the light budget of the laser scanning system, by perhaps enabling conventional light sources to be used. By replacing the point illumination and detection by a line-shaped

illumination and detection slits [4-6], acquisition speed was increased and the need to scan the sample was reduced to a single dimension. Another approach used a Nipkow wheel-type rotating disk to scan hundred or thousands of pinholes in parallel, the acquisition speed was tremendously increased at the expense of a lower sectioning capability [7, 8].

By exciting fluorescence emission only in the plane of interest with a thin light sheet perpendicularly to the detection, Selective Plane Illumination Microscopy (SPIM) [9, 10] provided inherent optical sectioning and proved particularly useful in developmental biology for high-resolution confocal imaging of large specimens such as embryos. Total Internal Reflection Fluorescence (TIRF) microscopy [11] made use of evanescent waves to excite fluorescence at the interface between a biological sample and a glass substrate. The superior axial resolution lead to unprecedented images of exocytosis processes at the cell membrane [12, 13].

A third concept soon emerged that took advantage of the increasing computer processing power and technological improvements in digital detector arrays, such as Charge Coupled Device (CCD) cameras. By patterning the illumination in a way that sharp intensity variations are present in only the focal plane of the microscope objective but attenuate with defocus, the confocal information can be coded and the out-of-focus blur can be removed using an appropriate post-processing algorithm in a computer. Several methods for generating the in-focus encoding pattern have been investigated.

The grid projection structured illumination technique [14-16] relies on a grid, which is introduced in the illumination path of a conventional microscope in order to project a modulation pattern onto the sample. This modulation pattern is shifted in steps of one third of a period whilst acquiring three images on an array detector. Calculating the root mean square intensity variation for each pixel across the three images constructs a sectioned image, whose axial resolution was shown to be better than better than the PSCM system [17]. A similar technique called aperture correlation microscopy [18] was implemented using a patterned disk with a high fill factor to generate the in-focus illumination and matched detection pattern.

On the other hand, Dynamic Speckle Illumination microscopy (DSI) [19-21] makes use of the granular intensity pattern resulting from a coherent illumination of the sample to generate a random in-focus structure that disappears with defocus when

imaging fluorescence light. The confocal image is calculated after acquiring several images and extracting the image from where the speckle pattern is seen to move on the object. These techniques rely on minor modifications of the conventional microscope system, are very cheap to implement, can be fast enough to produce real time confocal images, and in the case of the structured illumination and aperture correlation techniques, do not require laser light illumination.

Light emission has seen a revolution with the introduction of nitride based semi-conductor materials at the beginning of the 1990's. For the first time, intense quasi-monochromatic visible and UV light could be produced from a semi-conductor and a large spectrum of wavelengths from green to deep ultra-violet (UV) [22] was soon available by changing the semi-conductor alloy composition. Semi-conductor materials can be packaged into Light Emitting Diodes (LED), which have now become ubiquitous in our society. The Institute of Photonics in Strathclyde University in Glasgow initiated a Basic Technology Research program in 2003 that aimed at designing and manufacturing Gallium Nitride (GaN) LED micro-arrays using standard lithography techniques, whose elements are individually addressable [23-28]. The resulting micro-displays are fast, bright and the emission wavelength can now reach the deep UV. The project included other partners with expertise in areas ranging from materials growth, through packaging and micro-optics to end users, such as our own group, who would develop applications for these devices in the fields of microscopy, biochips and displays.

This thesis investigates the application of GaN micro-LED arrays as a primary solid-state illumination source for confocal imaging with no moving parts. A number of different techniques are developed, demonstrated and analysed, showing real potential for the technology, particularly where system miniaturisation and ruggedness might be important. As a second area of application the devices are used as patternable sources for optical neuro-stimulation where neuron activity is generated with high spatial and temporal resolution.

2. Thesis overview

Chapter 2 describes some of the relevant microscopy techniques that can currently be used to obtain sectioned images and the underlying principles of image formation are explained. It is first demonstrated how imaging in a conventional microscope does not

exhibit optical sectioning and then the theory of imaging in a point scanning confocal microscope is described to show how it does provide sectioning. The impact of various illumination and detection geometries on the ability of the microscope to discriminate depth is investigated and several widefield alternatives to point scanning confocal microscopy are described.

Chapter 3 introduces the micro-LED array technology and describes how this technology evolved from matrix arrays of moderately bright top-emitting emitters into a much brighter and more uniform matrix and stripe arrays of flip-chip micro-LEDs. The design and fabrication of electrical drivers used to display arbitrary patterns on the micro-LED arrays is key to enabling much of the later work described in this thesis and is presented here. In this section it is explained how the drivers compensate for some of the LED defects, how they allow for the reconfiguration of the light pattern within a few microseconds and how they communicate with the host computer where dedicated graphical interfaces facilitate straightforward manipulation of the light patterns.

The optical sectioning process of a structured illumination microscope is described in detail in Chapter 4 and the performance of such a system in presence of photon shot noise is investigated. Noise is a significant issue in any sectioning technique where the sectioned image is calculated by post-processing in a computer as the noise detected along with out-of-focus image components cannot be removed by the processing technique. Confirmation of this theory is demonstrated with conventional grid projection structured illumination microscope. Finally, the same technique is demonstrated using the novel micro-stripe LED array as a patterned light source as a complete system with no moving parts.

Keeping the microscope setup the same in Chapter 5, the micro-stripe array is used as a linear light source for line-scanning microscopy. The system performance is measured and the degradation in optical sectioning of the slit scanning system compared with a PSCM is investigated, both when illuminating the sample with single and multiple line patterns. An experimental method to subtract the remaining background light is introduced and experimental verifications of this method are presented, showing dramatic improvements in out-of-focus signal rejection.

In Chapter 6 the theory of improved optical sectioning in a line microscope via subtraction of the out-of-focus light is developed. The sectioning of the new

system is compared with the axial resolution of a PSCM and it is shown to reduce out-of-focus signal by almost an order of magnitude faster with defocus. Furthermore, theory for illuminating the specimen with a multiple line pattern is also developed and this leads to the design of a new double-sided spinning disk for high-speed confocal imaging having enhanced axial resolution.

Application of micro-structured LED arrays to neuroscience is described in Chapter 7 as a means of providing patterned illumination for photo-stimulation of neurons and neural networks. The possibility of using UV micro-emitters to realise the photolysis of caged fluorophores is demonstrated. Blue arrays are then used to spatially excite neurons that are rendered photo-sensitive to blue light after being transfected with a light activated membrane protein, Channelrhodopsin-2. The design of a novel system for multiple-stimulation multiple-detection of neuron networks is presented. This system should enable more precise in-vitro simulation of complex neural systems. Finally, a long-term project to realise non-invasive retina prosthesis is described, where the blue LED array is used as a light source for direct patterned excitation of human retinal ganglion cells.

Chapter 2 Introduction to widefield three dimensional microscopy techniques

When looking at a three-dimensional object, such as a cell or tissue, through the binocular of a conventional widefield microscope, it is clear that this instrument has no ability to discriminate depth. The light originating from the out-of-focus part of the sample produces a blurred image, which is mixed together with the in-focus information in the widefield image. Under normal circumstances, it is not possible to extract only the in-focus part of the object that contains the information of interest. Thus it is not unusual that in a conventional microscope image, more than 90% of the light is out-of-focus light that can obscure the in-focus details and reduce dramatically the image contrast. In other words, a conventional microscope cannot perform optical sectioning. A significant issue also arises from the scattered light, which can be diffracted, reflected and refracted within the specimen itself. Because imaging deeper and deeper into a sample increases the chances of scatter, the image captured by the conventional microscope will appear more and more blurry.

The aim of this chapter is to provide first a brief introduction of the image formation process in a conventional microscope. This is followed by a description of several techniques used to reconstruct a three dimensional image of an object. It begins with a short description of the principles and instrumentation of a confocal microscope, which is presently the most widely used system to achieve optical sectioning. Operation of the confocal imaging system in fluorescence is explained. This is followed by a brief description of several widefield techniques used to retrieve only the in-focus information in a fluorescence microscope, namely spinning disk microscopy, aperture correlation microscopy and dynamic speckle microscopy, all of which increase the illumination light budget compared with a single point scanning system, and Selective Plane Illumination Microscopy (SPIM) and Total Internal Reflection microscopy (TIRF), which allow for specifically exciting the fluorophore molecules in the planes containing the information of interest. Finally, several methods of improving the lateral and axial resolutions in a widefield fluorescence

imaging system are presented that rely on projecting a fine structure on the sample to observe spatial frequencies that are not captured by the objective lens.

1. Image formation in a conventional fluorescence microscope

1.1. Introduction

This section aims at describing the image formation in a conventional widefield microscope and explaining why this imaging system is unable to discriminate depth. Consider first an aberration-free incoherent imaging system, where an object that is either fluorescing or illuminated incoherently is imaged onto an infinite 2D detector. It is clear from the ray diagram depicted in Fig. 2.1 that all the planes located at different depths in the 3D object will contribute to the 2D image captured by the detector. The light originating from a single in-focus point located on the optic axis (P) will form a sharp spot onto the detector, whereas the light coming from the out-of-focus planes (P' and P'') will form blurred images having lower spatial frequency content. When looking at a 3D object, the blurred images coming from the out-of-focus planes will contribute to the final widefield image together with the in-focus information.

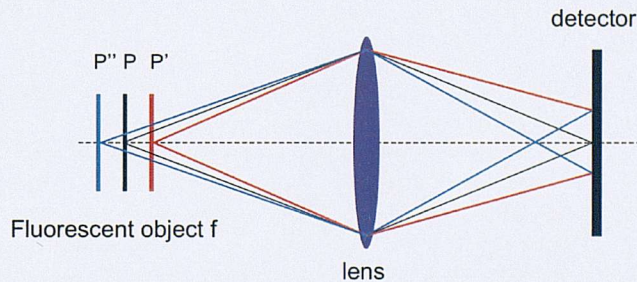


Fig. 2.1. Ray diagram illustrating the effect of focusing the light coming from out-of-focus planes (P' and P'') and an in-focus plane (P) onto an infinite 2D detector

This out-of-focus light does not carry any useful information, but only contributes to blurring the microscope image. The fundamental reason why it is impossible to discriminate depth with a conventional microscope is that the 3D specimen is imaged onto a 2D detector.

It is beyond the scope of this thesis to go into great details of the theory of image formation in a conventional widefield microscope. However, it is important to

understand a little theory about the inability of a conventional microscope to discriminate between in and out-of-focus light, and to derive its lateral and axial spatial resolution, which determines how small an object can be imaged accurately. The description will be limited to some elements of the paraxial theory for imaging fluorescent objects.

Normalised optical units (v,w,u) will be extensively used in the following chapters and are related to the real coordinates (x,y,z) via [2]:

$$\begin{aligned} (v,w) &= \frac{2\pi n}{\lambda} \sin(\alpha) (x,y) \\ u &= \frac{8\pi n}{\lambda} \sin^2(\alpha/2) z \end{aligned} \quad (2.1)$$

where λ denotes the wavelength of the light, $NA = n \sin(\alpha)$ is the numerical aperture of the lens and n is the refractive index of the object medium. Optical units are useful here as they make theoretical considerations independent from the microscope objective parameters. When working in a cylindrically symmetric coordinate system, the normalised radial optical coordinate will also be used.

$$r = \frac{2\pi n}{\lambda} \sqrt{x^2 + y^2} \sin(\alpha) \quad (2.2)$$

1.2. Image of a single point emitter

When a lens forms the image of a single bright point sitting in focus on the optic axis, the intensity in the image [2, 29] is given by Equation (2.3).

$$I(v,w) = |h(v,w)|^2 \quad (2.3)$$

where h is the amplitude point spread function of the lens, which describes how the phase and amplitude of the wavefront originating from a point source is transformed when passing through the imaging system.

$$h(v,w) = \iint P(x',y') \exp[j(vx' + wy')] dx' dy' \quad (2.4)$$

where P is the pupil function of the lens, which describes the physical aperture of the lens and its transmissivity, and x' and y' are normalised co-ordinates in the pupil plane, being of value 1 at the edge of the pupil. If the pupil is assumed to be circularly symmetric, Equation (2.3) can be written as

$$I(r) = |h(r)|^2 \quad (2.5)$$

where the circularly symmetric PSF is now given by:

$$h(r) = 2 \int_0^1 P(\rho) \rho J_0(r\rho) d\rho \quad (2.6)$$

J_0 is the zero order Bessel function of the first kind and ρ denotes the normalised radial coordinate in the pupil. In case of an ideal circularly symmetric aperture, the pupil function can be expressed as

$$P(\rho) = \begin{cases} 1 & \rho \leq 1 \\ 0 & \text{else} \end{cases} \quad (2.7)$$

The intensity in the image of a point source in focus thus simplifies into

$$I(r) = \left(\frac{2J_1(r)}{r} \right)^2 \quad (2.8)$$

which is the normal Airy Pattern. In order to analyse the response of the conventional microscope to defocus, defocus is introduced in the PSF as a quadratic phase factor in the pupil plane which scales with the defocus u

$$h(r, u) = 2 \int_0^1 P(\rho) \exp\left(\frac{1}{2} j u \rho^2\right) \rho J_0(r\rho) d\rho \quad (2.9)$$

In general this integral is difficult to evaluate analytically, although several series solution exist. However if the single point emitter is moved throughout the focus along the optic axis ($r=0$), then the integral can be evaluated simply to give the intensity of the detected signal as:

$$I(u) = \left[\frac{\sin(u/4)}{u/4} \right]^2 \quad (2.10)$$

1.3. Image of a defocused object

Consider now the image of a fluorescent object described by the fluorescence generation function $f(v, w, u)$. Because the imaging is incoherent, each point in the object will emit light with a random phase and the image on the detector will be the sum of the intensities of the points across the object convolved with the PSF of the system. The image of the object on the detector will then be linear in intensity [30] and can be written

$$I(v, w, u) = |h(v, w, u)|^2 \otimes f(v, w, u) \quad (2.11)$$

where $\otimes$ denotes the convolution operation.

In order to describe how the system transmits signals as of function of spatial frequency, it is common practice to define its behaviour in terms of transfer functions. The optical transfer function $T(s, u)$ is defined as the Fourier transform of the incoherent point spread function $|h(r, u)|^2$. The incoherent optical transfer function with defocus is a rather complicated function, which was analytically derived by Hopkins as an expansion of Bessel functions [31] and experimentally approximated by Stokseth for an aberration free low NA system [32]. The OTF represents how the different spatial frequencies in the object are attenuated by the imaging system at each depth. If the OTF is assumed to have cylindrical symmetry, the Stokseth approximation $T(s, u)$ is given by:

$$T(s, u) = \begin{cases} g(|s|) \frac{2J_1[us(1-|s|/2)]}{us(1-|s|/2)} & \text{if } 0 < |s| < 2 \\ 0 & \text{otherwise} \end{cases} \quad (2.12)$$

$$g(s) = 1 - 0.69s + 0.0076s^2 + 0.043s^3$$

where s represents now the normalised frequency associated with the radial coordinate and J_1 is the Bessel function of the first kind.

$$\begin{aligned} s_x &= \frac{\lambda}{n \sin \alpha} f_x \\ s_y &= \frac{\lambda}{n \sin \alpha} f_y \\ s &= \sqrt{s_x^2 + s_y^2} \end{aligned} \quad (2.13)$$

Note that the maximum value of s for which the OTF has a non-zero value is 2 and this represents a fundamental limit to the maximum spatial frequency that can be resolved by the system.

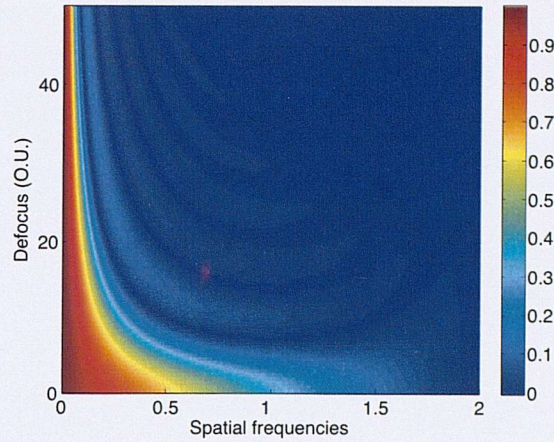


Fig. 2.2. Incoherent optical transfer function with defocus

Because the Stokseth approximation will be extensively used in this thesis when describing responses to defocus of different microscopes, more details of this approximation are given in Appendix 1.

It is interesting to look at the response of the system to defocus when imaging a thin fluorescent object $f(r,u) = \delta(u)$, where δ denotes the Dirac delta function. Considering the variation of total power in the image and from conservation of power, the response to defocus of the conventional microscope can be written [33]:

$$\begin{aligned} I_{conv}(u) &= \int_0^{\infty} \delta(u) \otimes |h(r,u)|^2 r dr \\ &= T(0,u) \end{aligned} \quad (2.14)$$

which is constant with defocus. This shows that a widefield microscope does not exhibit optical sectioning. If the specimen has no lateral spatial frequencies, the user will not be able to know at which depth the object is located. This is illustrated in Fig. 2.2, where the transfer function remains the same for all values of defocus when $s=0$.

1.4. The three dimensional Optical Transfer Function

Another way of describing the imaging properties of an incoherent lens system is to look at the OTF in three dimensions, which is the complete 3D Fourier transform of the incoherent PSF, instead of simply the OTF with defocus. The 3D OTF for small apertures was analytically determined by Frieden [34] and approximated by Gu and Sheppard [35], who simply performed the Fourier transform of Equation (2.12) with

respect to the variable u . Later the 3D OTF for a microscope with a high numerical aperture was calculated by Philip [36]. The 3D OTF is discussed here because it is a convenient way of visualising the imaging properties of different microscopes and comparing their performances.

The exact form of the 3D OTF $C(s, s_z)$ in the whole (s, s_z) frequency space can be written [35]:

$$C(s, s_z) = \frac{1}{|s|} \operatorname{Re} \left(\sqrt{1 - \left[\frac{|s_z|}{|s|} + \frac{|s|}{2} \right]^2} \right) \quad (2.15)$$

where s_z is the normalised axial spatial frequency given by

$$s_z = \frac{\lambda}{4n \sin^2(\alpha/2)} f_z \quad (2.16)$$

The complete 3D OTF is a solid of revolution about the v axis. Fig. 2.3 shows the OTF plotted in a plane parallel to the optic axis. Because of the discontinuity existing in $(0,0)$, $s \times C(s, s_z)$ is displayed here. The 3D OTF exhibits a missing cone of spatial frequencies around the origin (white lines), which suggests that the information in this region cannot be imaged.

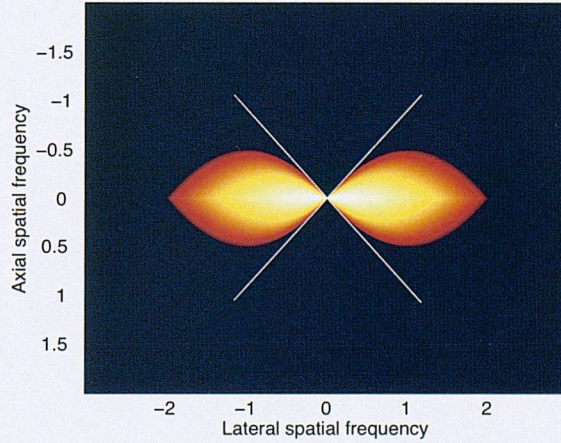


Fig. 2.3. Shape of $s \times OTF$ in a plane parallel to the optic axis

Achieving optical sectioning is then equivalent to filling the missing cone of the 3D OTF. Another important observation is that in the limit when $s_0 = 2$ (i.e. the finest grid that can be resolved by the lens) the extent of the 3D OTF along the v axis again becomes zero and here too it is difficult to localise very high spatial frequency structures in the axial direction.

2. Principle of confocal microscopy

The confocal microscope was invented by Marvin Minsky in 1961 [1] but remained unrecognized until the late 1970's when Egger and Davidovits [37], Sheppard and Wilson [33] and Brakenhoff [38] developed the same concept. Minsky realised that by illuminating the object with the image of a point light source and detecting the light from the object using an aligned point detector it was possible to reject most of the out-of-focus light, achieving what we now refer to as the confocal effect. The object was then built up by scanning the object across the projected point of light to produce an image. Confocal microscopy has since evolved from a complex instrument into a commercial product and has become a standard tool for modern biological research thanks to the improvements of digital detectors and beam scanning units, and the wide availability of laser sources. This section describes the basic operation of a confocal microscope and discusses different detector geometries.

2.1. Basic principles

2.1.1 *Microscope setup*

Transmission and reflection type confocal microscopes are depicted in Fig. 2.4 (a) and (b), respectively.

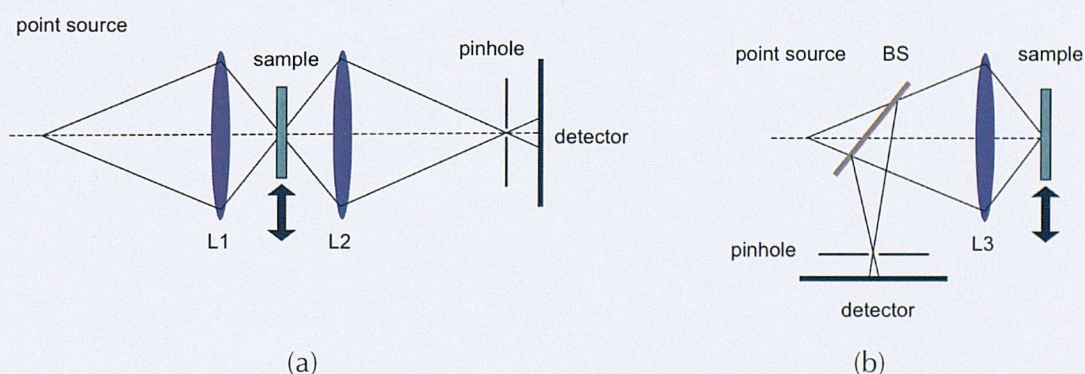


Fig. 2.4. Confocal microscope setups to achieve optical sectioning (a) Transmission type confocal system. (b) Reflection type confocal system. BS: Beam Splitter.

In a transmission microscope, the illumination lens L1 images a point source (typically a laser beam) onto an object, whose transmittance varies from point to point. The light transmitted through the sample is focused by the detection lens L2 onto a pinhole and measured by a photo-detector. In the reflection mode, illumination

and detection take place through the same objective lens. The reflected signal is also filtered by a pinhole after been reflected towards the detector using a beam-splitter.

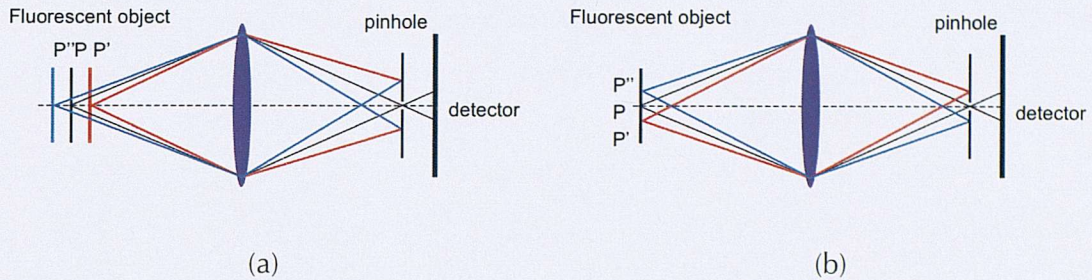


Fig. 2.5. Ray diagrams of a confocal detection illustrating the rejection of the light coming from (a) the out-of-focus planes and (b) the off-axis points in the focal plane

The role of the detection pinhole in rejecting the out-of-focus light can be illustrated with simple ray diagrams similar to those shown previously in Fig. 2.1. As shown in Fig. 2.5 (a), light emitted on-axis from the in-focus plane (P) passes through the pinhole and reaches the detector, while the pinhole blocks most of the light originating from the out-of-focus planes (P' and P''). Light emitted off-axis is also blocked by the pinhole (Fig. 2.5 (b)), which permits the process to define a true 3D confocal volume. In order to build a confocal image the sample has to be scanned across the light beam. Alternatively, beam scanning units composed of two mirrors scanning the beam in the lateral directions can also be used, which has the advantage of keeping the sample still during acquisition and avoid mechanical disturbance.

Illuminating the sample with a point source, as shown in Fig. 2.4, enhances the confocal effect and increases both axial and lateral resolutions of the microscope. The size of the detection pinhole was shown to be optimal when infinitely small [39, 40] and the performance gradually degrades with increase in the pinhole size. In practice, the pinhole is set large enough to detect sufficient signal, at the expense of a slightly reduced confocal effect. If the pinhole is smaller than one Airy disc the sectioning capability of the microscope is almost not degraded.

2.1.2 Image of a point emitter in a confocal microscope

The performance of a confocal system has been described by Wilson and Sheppard [2]. They showed that the confocal PSF is the product of the illumination and

detection PSF. Thus, the intensity in the image of a single point in a confocal system is given by:

$$I(r, u) = |h(r, u)|^4 \quad (2.17)$$

where h is the amplitude PSF of an aberration free in focus system having the same illumination and detection lenses, such as the imaging system depicted in Fig. 2.4 (b). The intensity in the image of a single point is given by:

$$I_{conf}(r) = \left(2 \frac{J_1(r)}{r} \right)^4 \quad (2.18)$$

The point spread functions of a conventional and confocal microscopes are plotted in Fig. 2.6. The central peak of the confocal response is sharpened by a factor of 1.4 relative to the conventional response, which suggests an improvement in the resolving power of the confocal system.

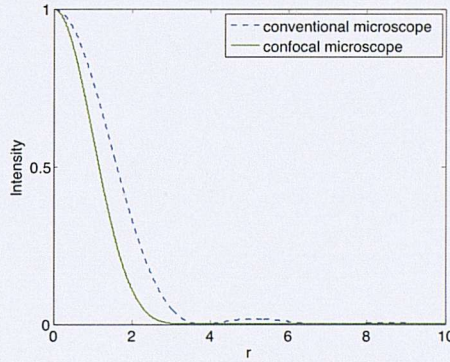


Fig. 2.6. Image of a single point object in a conventional and confocal microscope

Likewise, if a fluorescent point object is now moved along the optic axis, the confocal signal detected is now given by

$$I(u) = \left[\frac{\sin(u/4)}{u/4} \right]^4 \quad (2.19)$$

2.1.3 Sectioning strength

When scanning a planar fluorescent sheet object $f(r, u) = \delta(u)$, through the focus of a confocal system, the integrated intensity of the confocal system with defocus will be:

$$\begin{aligned} I_{conf}(u) &= \int_0^\infty \delta(u) \otimes |h(r, u)|^4 r dr \\ &= \int_0^\infty T^2(s, u) s ds \end{aligned} \quad (2.20)$$

which is plotted in Fig. 2.7. together with the conventional response to defocus. The confocal microscope clearly exhibits the ability to resolve the position of the sheet object in depth, ie it exhibits optical sectioning.

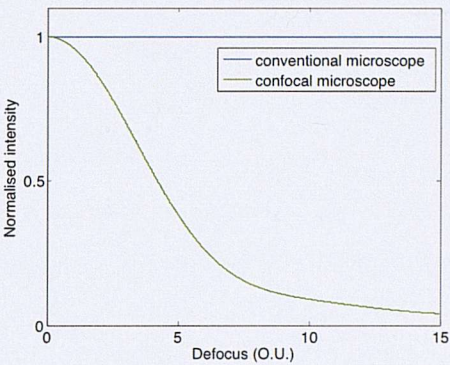


Fig. 2.7. Variation of integrated intensity with defocus for a conventional and a confocal microscope

2.1.4 Confocal resolution in terms of optical transfer function

From Equation (2.17) it is clear that the OTF of a fluorescence confocal microscope having the same illumination and detection lenses will be given by the convolution of the conventional OTF with itself. The confocal OTF is shown in Fig. 2.8, which is obtained by calculating the auto-convolution of the incoherent OTF. It can be observed that the missing cone is filled, which suggests that the confocal microscope can now resolve large features in depth and thus exhibits optical sectioning. Also the lateral frequency extent of the OTF is two fold the extent of the conventional OTF, which indicates a lateral resolution improvement by a factor of two in spatial frequency compared to the conventional system.

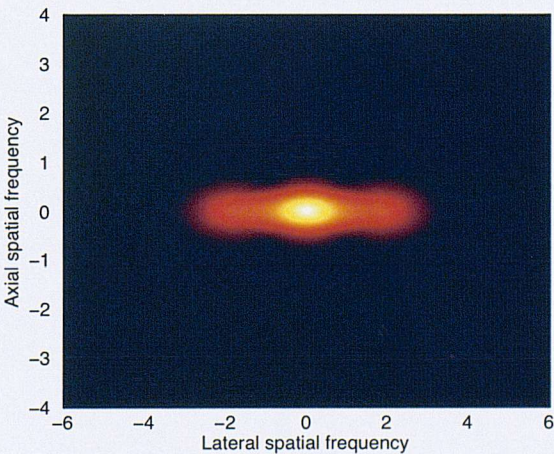


Fig. 2.8. Support of the confocal OTF

2.2. Confocal microscope operation in fluorescence

The use of fluorescence in biological science has known a remarkable growth during the last two decades and fluorescence is now a dominant methodology used in flow cytometry, DNA sequencing, medical diagnosis and genetic analysis. Because fluorescence labelling is highly selective, many developments have been made utilising fluorescence spectroscopy and microscopy in quest for optically obtaining more information from the sample of interest. Advances in laser technology, high speed electronics and computing power have induced the most dramatic growth in the use fluorescence in cellular and molecular imaging [41]. Combining fluorescence selectivity with the confocal optical sectioning property, confocal fluorescence microscopy has become a standard tool in life science for resolving molecular organisation. Covering all the areas of application of fluorescence microscopy is beyond the scope of this thesis. This section only attempts to briefly explain the phenomenon of fluorescence and its relevant applications for this work.

2.2.1 Fluorescence emission

Fluorescence is a process in which a molecule absorbs an incident photon, is excited into a more energetic state, and then spontaneously re-emits a fluorescence photon when relaxing from the excited state to the ground state. In highly conjugated organic molecules, the electronic molecular structure usually results in an efficient absorption in the visible part of the spectrum.

The fluorescence process can be illustrated with the use of a simplified Jablonski diagram (Fig. 2.9). This diagram describes the energy states of a fluorescent molecule along with the energetic transitions that can occur. The ground electronic state is denoted S_0 and the first excited state S_1 . Each energy level is sub-divided into vibrational energy levels of the fluorophore that are populated according to the thermal energy of the system. When absorbing an incident photon, the molecule is excited from the ground state to an energy level of one of the excited states. Once excited, the fluorophore populates one of the vibrational levels of the electronic states. It then relaxes to equilibrium through vibrational relaxations, within a time of typically 10^{-12} s. Over a period of typically 10^{-9} s in the excited state, known as the fluorescence lifetime of the fluorophore, the molecule undergoes spontaneous emission back to one of the vibrational levels of the ground state S_0 . Because of the loss in energy

involved in the thermal relaxation process, the wavelength of emission is in general longer than the wavelength of excitation. This phenomenon is known as the Stokes' shift [42].

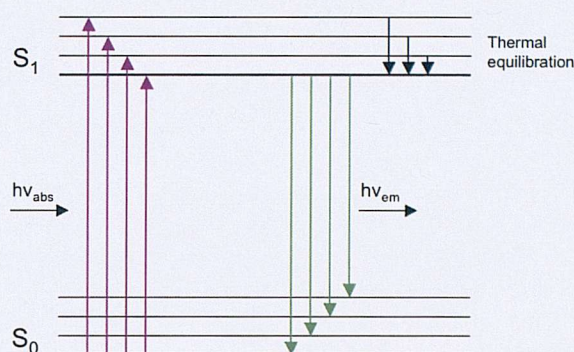


Fig. 2.9. Simplified Jablonski diagram describing the single photon absorption and fluorescence emission

Molecules of interest in a sample can be tagged using natural or engineered fluorescent markers (or dyes) that exhibit a high quantum yield. The sample is then excited and fluorescence is detected at a different wavelength using a dichroic filter. Thus, fluorescence emission in a microscope image provides an accurate location of the targeted molecule. More properties of the fluorophore emission can also be measured to provide more information about the physical and chemical environment of the molecule.

A change in the molecule fluorescence lifetime will indicate for instance a change in the energy level structure (through the excitation and emission spectrum), the surrounding local refractive index [43, 44] or a change in many environmental factors that affect the relaxation of the fluorophore excited state without emission of a photon. Hence, fluorescence lifetime measurements can be used to probe pH or solvent viscosity [45-47], for instance. Fluorescence emission can also be polarised following polarised excitation. The resulting fluorescence anisotropy describes this polarisation of the fluorescence emission and can be used to determine the orientation of the fluorescent molecules, such as the orientation of fluorescent protein in cell membranes [48, 49]. Combining fluorescence anisotropy with fluorescence lifetime measurements allows measurement of the rotational mobility of a fluorophore and therefore the local viscosity [50].

2.2.2 *Fluorescence confocal microscopy*

When operating in fluorescence, the excitation light is focused onto the specimen and the fluorescent emission is separated from the excitation by a dichroic mirror and an emission filter (Fig. 2.10). As before, light emitted from the location of the excitation spot goes through the pinhole in front of the detector.

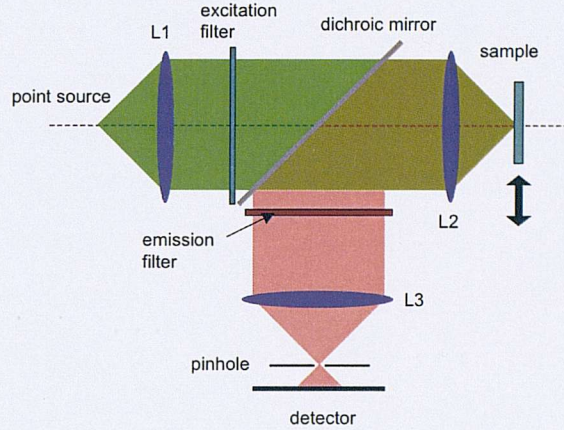


Fig. 2.10. Layout of the confocal microscope operating in fluorescence

In the theory of confocal imaging described earlier, the excitation wavelength was assumed to be the same as the detection wavelength. In the case of a confocal microscope imaging a fluorescent specimen, the emission wavelength is a factor $\beta = \lambda_{em}/\lambda_{ex}$ longer than the excitation wavelength. The image intensity can then be written

$$I = |h_{eff}|^2 \otimes f \quad (2.21)$$

where h_{eff} is given by

$$h_{eff} = h(v, u)h(v/\beta, u/\beta) \quad (2.22)$$

In order to excite the fluorophore with maximum efficiency and detect the maximum amount of fluorescent signal, λ_{ex} and λ_{em} are usually chosen close to the maxima of the excitation and emission spectra, separated from each other by the Stokes' shift. The dependence of the 3D optical transfer function on the pinhole radius and emission wavelength was studied [40] showing that as the Stokes' shift increases, the 3D OTF shrinks and missing data appears on the frequency axis in the optical axis direction. The OTF eventually converges towards the transfer function of a conventional microscope when the fluorescence wavelength becomes much longer than the excitation wavelength.

2.3. Slit scanning confocal microscope

The superior resolution and optical sectioning property in a confocal microscope result from the use of a detection pinhole to block the out-of-focus light and sharpen the microscope PSF. The pinhole detector, however, results in a decrease in the measured signal. It also produces an image signal from only one point of the object at a time, so that in order to build up a complete image, some form of scanning is necessary. Different illumination and detection geometries have been investigated with the aim of releasing the need for both an expensive scanning system and a bright laser light source. Using slit illumination and detection rather than pinholes alleviates the necessity for 2D beam scanning and dramatically improves the light throughput of the system at the expense of degrading the optical sectioning property of the microscope.

In a slit-scanning confocal microscope, the point source is replaced by a line illumination usually obtained by focusing a laser beam onto the specimen with a cylindrical lens and the detection pinhole is replaced by a detection slit. Thus, the illumination only needs to be scanned in the dimension perpendicular with the line axis. Let's assume that a line illumination parallel to the w -axis is projected onto a fluorescent sample $f(v, w, u)$. Neglecting the Stokes' shift and assuming detection along the same w -axis, the intensity in the image is given by

$$\begin{aligned} I(v, w, u) &= [PSF_{ill}(v, w, u) \times PSF_{det}(v, w, u)] \otimes f(v, w, u) \\ &= |h(v, 0, u)|^4 \otimes f(v, w, u) \end{aligned} \quad (2.23)$$

Image formation can be shown to be similar to a conventional microscope and point scanning confocal microscope in the directions parallel and perpendicular to the slits, respectively [5, 51, 52]. If a fluorescent sheet object is scanned through the focus of the objective and using the same notations as before, the integrated response to defocus of a line-scanning microscope can be written

$$\begin{aligned} I(u) &= \int_0^\infty \delta(u) \otimes |h(v, u)|^4 dv \\ &= \int_0^2 T^2(s, u) ds \end{aligned} \quad (2.24)$$

The response to defocus of the point and slit scanning microscopes are plotted in Fig. 2.11. It is clear that the ability of the slit microscope to reject out-of-focus

background is degraded compared to the point scanning system. In the case of fluorescence imaging, the full width at half maximum (FWHM) of the slit system is increased by a factor of approximately 1.6 in the axial direction.

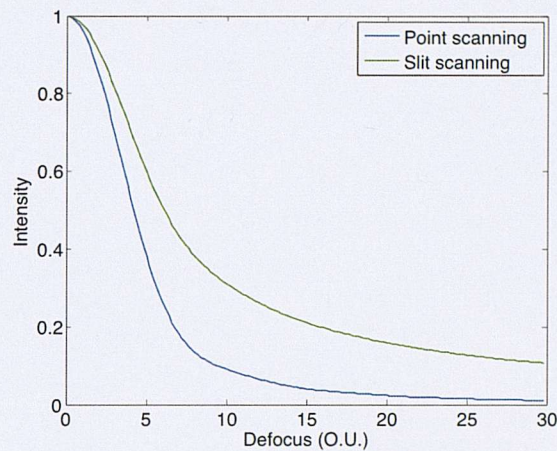


Fig. 2.11. Microscope response to defocus when illuminating the sample and detecting the fluorescence light with a point and a slit

However, the line scanning system has advantages in terms of instrumentation complexity and acquisition speed. Because in a fast scanning system the fundamental limit to signal is usually set by the saturation of the fluorophore, line-scanning systems perform an order of magnitude better than the point scanning with respect to the detected signal level [53]. Thus, the line-scanning microscope has some advantages for video-rate imaging, especially when using resonant scanners or acousto-optic deflectors to scan the beam on the specimen [54]. A commercially available line microscope was released recently that captures 512x512 confocal images in the excess of 100 frames per second [55].

3. Techniques to achieve optical sectioning in a widefield microscope

The confocal technique has triggered a revolution in imaging of biological specimen but suffers from an inherently low speed as a single spot needs to be scanned across the entire field of view. Many techniques have been developed in order to achieve optical sectioning either by increasing the confocal speed or by generating fluorescence emission in a specific plane of interest while acquiring a widefield image. This section presents those two concepts.

3.1. Nipkow disk microscope

A method to increase the scan speed of a confocal microscope is to parallelise the imaging by creating a multipoint excitation/detection scheme. The first microscope operating on this principle was developed by Petran in 1968 [7] and was based on a Nipkow disk. This disk consists of an array of pinholes through which the excitation light passes Fig. 2.12. The pinholes are imaged onto the specimen. The same set of pinholes provides the sectioning for the fluorescence light. By spinning the disk at high speed, the spot pattern is scanned over the specimen and a confocal image is captured by the CCD camera. More recently, the use of a lenslet array to focus laser light into the pinholes has solved the problem of excitation light efficiency.

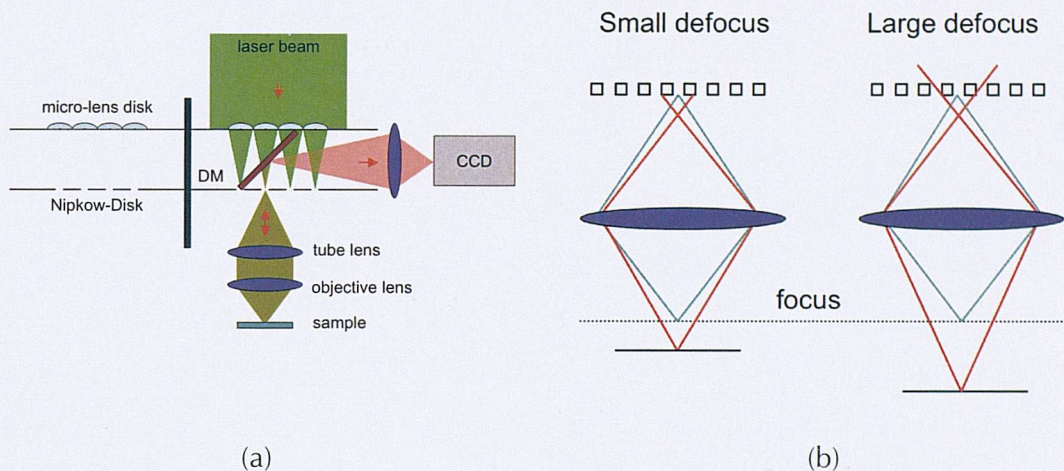


Fig. 2.12. Nipkow confocal scanner: (a) microscope setup and (b) ray-diagram of the rejection of out-of-focus light in presence of small and large defocus

The impact of multiple imaging channels can be gauged when looking at how light from different planes passes through the optical system. In Fig. 2.12 (b), a point in the focal plane is both illuminated and imaged through the same pinhole. In contrast, light from below the focal plane is blocked by the pinhole disc. This is analogous to the situation in a single beam microscope. Nonetheless, at a certain distance from the focal plane, light will begin to pass through the adjacent holes, and will appear in the final image. Thus, the pinhole size and the distance between pinholes must be selected carefully in order to avoid this cross talk and subsequent loss in confocal sectioning [56, 57]. A second benefit of the parallel illumination system is that the spinning disk produces a very low level of peak intensity in the illumination light,

which has clear advantages in terms of fluorophore saturation and photo-bleaching rate [58, 59].

3.2. Optical sectioning by aperture correlation / Programmable Array Microscope (PAM)

Several microscope developments aimed at increasing the density of pinholes in a Nipkow disk type system in order to increase the light budget and remove the need for laser illumination. However, as the distance between pinholes decreases, cross talk becomes a significant issue and eventually results in a background offset and a loss in confocal sectioning. The concern about removing this out-of-focus background led to two similar techniques that use a programmable diffractive device to generate the illumination pattern. The first method called aperture correlation was published by Wilson and al. [18, 60] and used a rotating disk to as the aperture mask. The basic is shown in Fig. 2.13. The aperture mask is imaged onto a specimen and light reflected back from the specimen passes back through the mask. A CCD camera that is focused onto the mask captures the image. As the disk is rotated, the confocal image builds up on the detector together with a non-confocal offset, due to the crosstalk. A composite image is thus obtained which consists in a confocal image superimposed upon a non-confocal image. It is then necessary to capture an additional widefield image to extract the confocal information.

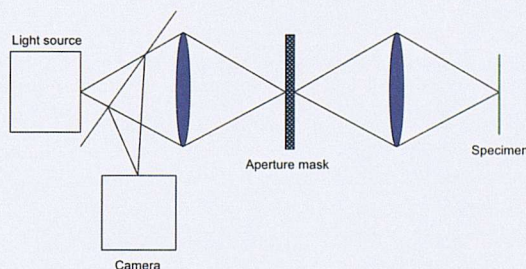


Fig. 2.13. Schematic diagram of the confocal microscope. The mask is imaged onto the sample and the fluorescence light is passed through the same mask to form the confocal image.

The same idea was implemented using a digital micro-mirror device (DMD) where the confocal and non-confocal images are captured by the same CCD detector before the latter is subtracted from the composite image [61-63]. This technique called programmable array microscope (PAM) allows for rapid optical sectioning with no moving parts but suffers from light inefficiency due to a high level of diffraction.

3.3. Dynamic speckle microscopy

The use of coherent speckle patterns has also been suggested to achieve optical sectioning in a conventional fluorescence microscope [19, 20, 64]. Under coherent widefield laser illumination of a scattering or diffusing plate, the summation of random optical phases gives rise to a fine speckle structure in the resultant optical field. This is usually avoided in illumination systems as it degrades the microscope image. However, speckle illumination can induce granularity in the fluorescence image that appears highly contrasted at the focal plane but blurred elsewhere (Fig. 2.14). A random shift in the speckle pattern thus leads to a large sharp variation in the in-focus light but to a small variation in the blurred out-of-focus light.

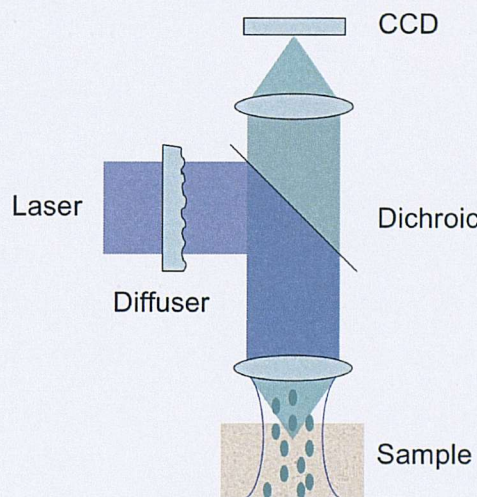


Fig. 2.14. Setup for dynamic speckle microscopy

Several methods have been used to reconstruct the confocal image. In one arrangement 128 images were acquired with random speckle illumination and the root mean square was calculated for each pixel [19] across the data set. Alternatively, the speckle pattern can be translated and an algorithm based on the intensity variation between two frames is used to reconstruct the confocal image [20]. This processing improves the optical sectioning ability of the system as successive images retain the out-of-focus correlations, and so a fairly accurate estimate of the out-of-focus light is easily measured before subtraction. This method has been recently improved by wavelet pre-filtering the images before processing the rms values of the dataset [21]. This processing helps removing the out-of-focus uniform blur and leads to the same results as the previous method with no need for translating the illumination pattern.

Dynamic Speckle illumination is a robust and cheap way of obtaining optical sectioning in a conventional fluorescence microscope. However, it suffers from inherent drawbacks. First, a large number of images need to be acquired, which prevents the recording of any transient phenomena. Another drawback is its inherently degraded sectioning capability compared with a confocal microscope. This is due to the speckle illumination pattern having a very large depth of field. The sectioning process relies on only the incoherent detection of the emitted fluorescence, whereas both the illumination and detection pathways contribute to increasing the sectioning capability in a point scanning confocal system. As a result the performance of this microscope with point objects is identical to that of a normal widefield microscope. This same infinite depth of field prevents any use of the technique in reflection mode, unless interferometric detection is used [65].

3.4. Selective plane illumination microscopy (SPIM)

Selective Plane Illumination Microscopy is a widefield imaging technique used to achieve optical sectioning in thick, quasi-transparent specimens. In SPIM, a diffraction limited laser beam is focussed through the middle of a specimen using a cylindrical lens, generating a sheet of light that excites fluorescence from a single plane. An objective is placed a right angles to the light sheet to image the optically sectioned fluorescence on a wide-field detector, typically a CCD camera.

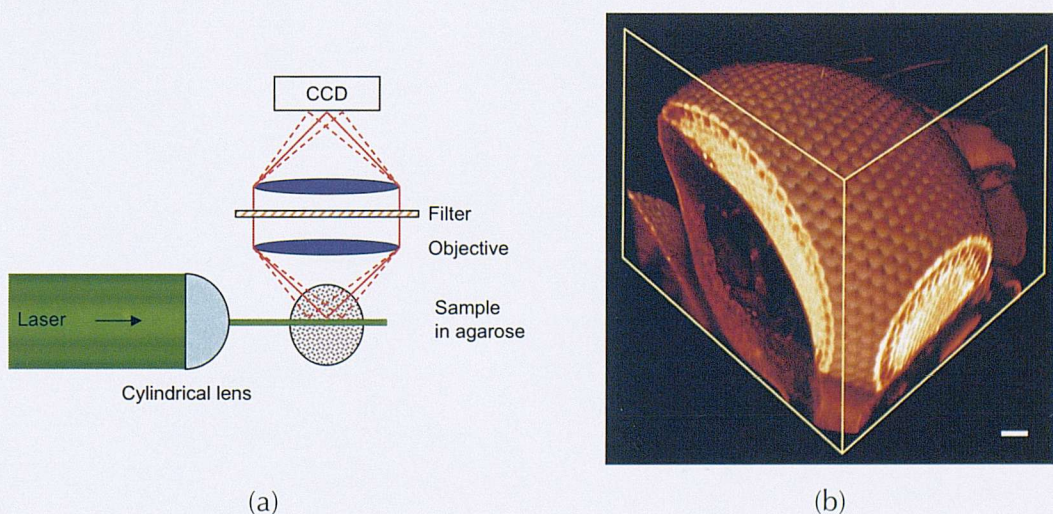


Fig. 2.15. SPIM microscopy: (a) Microscope setup and (b) auto-fluorescence image of a part of the faceted eye of a fruit fly captured with a 20x microscope [10]. Scale bar is 20 microns.

Two methods of generating the 3D data set can be used from this point. The first method consists in rotating the sample through 360° and capturing subsequent images at each angular position [9]. Deconvolution algorithms can also be employed to improve the image quality [66]. This method has the advantage of resulting in an isotropic resolution across the specimen, but this goes at the expense of intensive computation. The second method, also called ultra-microscopy, consists of illuminating the sample from the side and stepping the sample through illumination plane [10].

The axial resolution is thus limited by the width of the light sheet, while the lateral resolution is limited by the low NA of the imaging optics. The advantages of the SPIM system are that fluorescence originates only from the focal plane and large specimens can be imaged with micron resolution. It is well suited for developmental biology studies of embryos embedded in a low concentration agarose matrix, and in particular for the visualisation of high-resolution gene and protein expression patterns in three dimensions.

3.5. Total internal reflection microscopy (TIRF)

A beam, travelling in a medium of refractive index n_1 , which is incident on the interface with a medium of index n_2 at an angle θ , will undergo refraction according to Snell's law

$$\begin{aligned} n_1 \sin(\theta_1) &= n_2 \sin(\theta_2) \\ \theta_c &= \arcsin\left(\frac{n_2}{n_1}\right) \end{aligned} \quad (2.25)$$

If θ_2 reaches $\pi/2$ the beam will be totally internally reflected. This occurs at the critical angle θ_c . At incidence angles greater than θ_c , it is impossible to satisfy the boundary conditions at the interface by only considering the incident and reflected waves. Instead, it is found that a third evanescent wave is present in the lower refractive index material.

The TIRF microscope is a system that makes use of this principle to image the interfacial layer of the sample adjacent to the cover glass with extremely efficient reduction of the background fluorescence [12]. The high-energy excitation beam is totally internally reflected from the glass-water interface and generates an evanescent

wave that penetrates around 100nm into the sample. This illustrated in Fig. 2.16, where the excitation beam is represented by the solid and dotted blue lines.

There is very little background light because deeper focal planes are not excited. Changing the incident angle can control the depth over which the evanescent wave propagates. When the dotted excitation is used in Fig. 2.16, the evanescent wave will penetrate deeper into the sample, because of the smaller angle θ at which the beam hit the coverslip. This makes TIRF ideal for imaging single molecules and faint structures at this interface, such as exocytosis events [13]. The evanescent wave excites the fluorophore molecule and the emitted fluorescence light is collected in the same way as for the epi-fluorescence system.

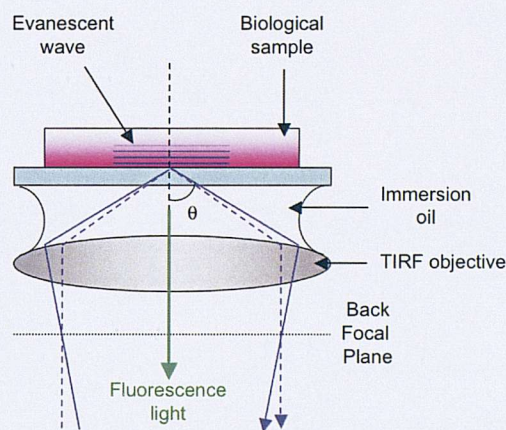


Fig. 2.16. Illustration of the microscope-sample interface in a TIRF system

Fluorescence intensity images of Human Embryonic Kidney (HEK) cells stained with di-4-ANEPPDHQ, which incorporates itself into the cell lipid bilayers where it becomes fluorescent, are shown in Fig. 2.17. The endoplasmic reticulum highlighted in the image of the equatorial plane gives an out-of-focus background in the widefield image of the interfacial layer (Fig. 2.17 (a)). This background light is not present in the TIRF image because no fluorescence is excited far above the surface of the coverslip.

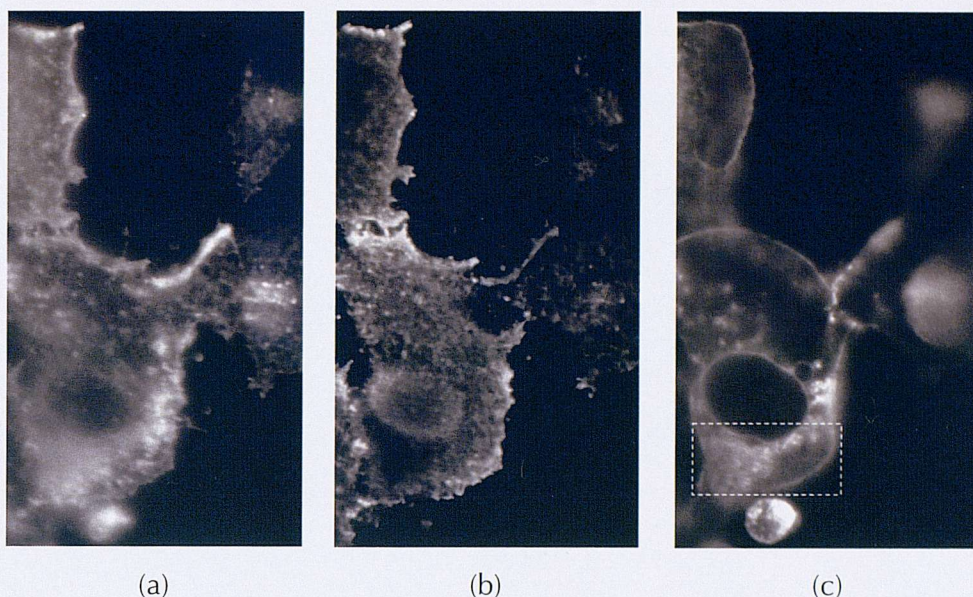


Fig. 2.17. Images of an HEK cell: (a) widefield image of the interfacial layer (b) TIRF image and (c) widefield image of the equatorial plane.

4. Linear axial and lateral super-resolution in a fluorescence microscope using patterned excitation

Even if the classical resolution limits are imposed by the imaging conditions, they can be exceeded as the limitations are only true under certain assumptions. In an imaging system, these assumptions are that observations take place in a conventional geometry in which the light is collected by a single objective lens and that the illumination is uniform across the field of view. Negating these assumptions offers several ways of overcoming the classical diffraction limit. First, by using excitation light that varies with position, higher sample spatial frequencies that cannot be directly imaged are captured by the lens in an indirect manner. Second, by gathering light over a larger set of angles around the sample using opposite objectives, enables increasing the overall NA of the imaging system.

4.1.1 *Resolution improvement by patterned excitation*

By exciting the sample fluorescence with a fine patterned structure, one can generate Moire patterns (Fig. 2.18 (a)) that can be used to increase the lateral resolution in a conventional widefield microscope. Assuming a one-dimensional grid pattern

$I_1(v) = I_1 \sin(\xi_1 v)$ of frequency ξ_1 illuminated with another grid pattern $I_2(v) = I_2 \sin(\xi_2 v)$ of frequency ξ_2 , the resulting grid image $I(v)$ can be written:

$$\begin{aligned} I(v) &= I_1 \sin(\xi_1 v) \times I_2 \sin(\xi_2 v) \\ &= \frac{1}{2} I_1 I_2 \left(\cos((\xi_1 - \xi_2)v) - \cos((\xi_1 + \xi_2)v) \right) \end{aligned} \quad (2.26)$$

The image is then composed of a lower difference frequency component and a higher sum frequency component. The highest frequency component ξ_1 might be filtered out during the imaging process, but by observing the difference frequency component and the knowledge of only the illumination spatial frequency ξ_2 , the information at spatial frequency ξ_1 can still be retrieved.

This principle can be applied to microscopy because the image of any object can be Fourier decomposed into an infinite series of sinusoidal patterns having different amplitudes, frequencies and orientations. By illuminating the specimen with a grid pattern translated and rotated in several positions, lateral resolution that exceeds the classical diffraction limit of a widefield fluorescence microscope. With this method the maximum detectable spatial frequency can be increased from ξ_2 to $\xi_2 + \xi_1$. Because the grid itself cannot be made smaller than the resolution limit s_0 , the highest spatial frequencies that can be detected is increased to $\xi_{2\max} = 2s_0$, twice the initial resolution limit (i.e. resolution is now the same as in a point scanning confocal microscope) [67].

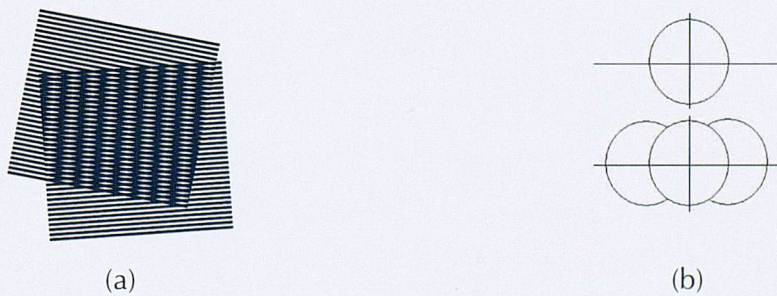


Fig. 2.18. (a) Moiré fringes. (b) Optical transfer functions (OTF) of microscope. The circles represent the microscope lateral cut-off frequency for uniform (above) and grid-projection (below) illumination. The extend of the frequency cut-off is shown to be twice as big in the latter case.

It is possible to remove the grid pattern in the recorded fluorescence image by some mathematical post-processing and additional image recording. A minimum of

three images with different grid phases are required to remove the grid pattern from the image. The obtained image will exhibit an improved resolution in the direction perpendicular to the grid. To improve the resolution in other directions, the same phase shifting procedure should be repeated at several angles. The same principle was recently used to double both the axial and lateral resolutions [68].

Further, exploiting the non-linearity properties of fluorophore saturation theoretically an unlimited improvement is possible [69]. However, in reality the improvement is limited by the signal-to-noise ratio and photo bleaching of the imaged fluorophores. Furthermore, the large number of images required for the image reconstruction prevents any use of this technique to live imaging.

4.1.2 Axial resolution improvement

Using two opposite microscope objectives, the concept of standing wave excitation was introduced that enables a significant improvement of the axial resolution [70]. Under opposite laser illuminations, the collimated coherent light produces an axial fringe pattern that excites an axially modulated fluorescence in the sample. This technique was recently shown to provide measurements of point-like objects with nanometre axial resolution [71].

5. Multi-photon fluorescence microscopy

Traditional confocal microscopy techniques, which use linear fluorescence processes, are usually limited to use near the tissue surface for high resolution imaging because at greater depths scattering tends to blur the image and degrades the sectioning capability [72]. In a linear excitation imaging technique, the fluorescence intensity of an emitter is linearly proportional to the intensity of the incident excitation light. On the other hand, multi-photon fluorescence microscopy techniques rely on the absorption of more than one photon for exciting the fluorescence contrast.

Most widely used in biological imaging is the excitation of fluorescence by two-photon absorption. Two photons than arrive within 0.5fs at a molecule can combine their energy to excite an electron from the ground state S_0 to the excited state S_1 , where the normal spontaneous fluorescence emission process can then take place [73], as illustrated in Fig. 2.19. Similarly three and more photons can combine together to excite fluorescence.

The efficiency of the two-photon absorption depends on physical properties of the molecule to fluoresce, through the two-photon absorption cross-section, and on the spatial and temporal properties of the exciting beam. Most of the non-linear processes have in common that they occur with very low probability under normal light conditions. Thus, the two-photon excitation has to be concentrated in space and time in order to generate sufficient fluorescence signal, making high NA objectives and ultra-short laser pulses a requirement to generate high peak intensities in the vicinity of the focal spot.

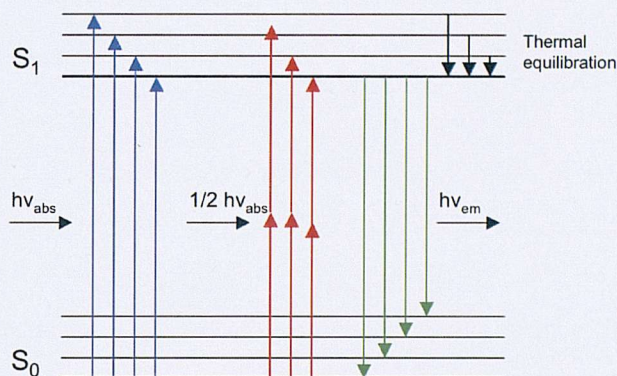


Fig. 2.19. Jablowski diagram illustrating the two-photon absorption process

Clear advantages have made the two-photon microscope a routine instrument for microscopic imaging of tissues. First, non linear signal production ensures that the fluorescence emission is localised in the vicinity of the focal spot, which thus results in an inherent optical sectioning ability with no need for spatially resolved detection through a detection pinhole, as shown in Fig. 2.20. As the localisation of the excitation is maintained even in strongly scattering media, two-photon microscopy is far less sensitive to scattering than traditional confocal microscopy techniques. The detection strategy then relies on collecting as many photons as possible and deep (up to 800 microns) imaging of tissues can be realised. Two photon microscopy has proven a particularly powerful tool for neural tissue imaging with micrometer resolution [74, 75].

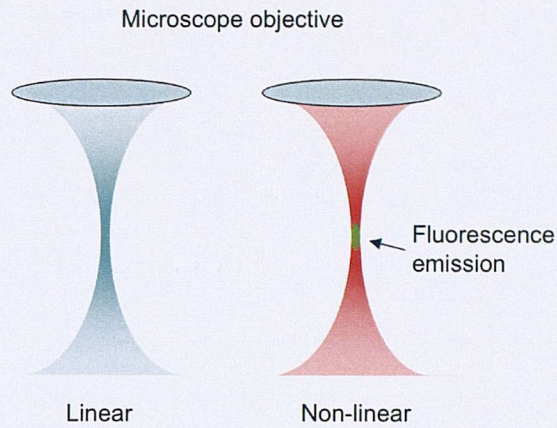


Fig. 2.20. Confinement of the fluorescence emission in a confocal and a two-photon microscope

The two-photon approach to optical sectioning does not make use of a spatially coded detection as for the microscopy techniques described in the previous section, but on the physical properties of both the fluorescent molecule and the excitation light. Reconstructing a sectioned image of a fluorescent object still requires scanning the excitation beam across the specimen, which usually limits the imaging speed. Strategies for scanning multiples laser beams on the sample have been developed that allow for real time two-photon imaging [76, 77] and produce widefield sectioned imaging at high frame rate.

6. Summary

Image formation in a conventional microscope was presented and its inherent lack of optical sectioning was explained both in terms of point spread and optical transfer functions. The confocal microscopy technique was explained where out of focus light is blocked by a pinhole detector, resulting in both increased image contrast and improved the resolution. Finally, methods to achieve optical sectioning in a widefield microscope were reviewed. Those methods all rely on an extra piece of information to fill in the missing cone of the conventional optical transfer function, either by spatially coding the light emission from the focal plane or by generating the fluorescence emission solely in a specific plane of interest.

Chapter 3 Micro-structured light emitting diodes and drivers

Light Emitting Diodes (LEDs) have emerged as very promising light sources, increasing in brightness from the dim photo-luminescent devices in the late 1960's to the current high brightness LEDs, which have become bright enough to replace incandescent lamps in many advanced microscopy techniques [78-80]. In fluorescence microscopy, where the quantum yield of fluorescent species is often very low and hence source brightness is critical, arc lamps, such as Mercury or Xenon burners, are usually required. These bulky, inefficient light sources generate a lot of heat, have a limited lifetime, cannot be controlled electronically and require filters to select the spectral bandwidth of the fluorescence excitation light. Conversely LED devices are usually very compact, can be modulated or pulsed and thus can be used in fluorescence lifetime measurement systems [81-83]. They have a long lifespan, are far more energy efficient and are relatively cheap. Additionally, the LED emission spectrum is narrow and the output wavelength has decreased from the mid-infrared in the 1960's to deep UV recently [84], making bright light sources commercially available at all wavelengths across the visible spectrum from 950nm down to currently 237nm.

In addition to their advantageous thermal, spectral and temporal features, conventional semiconductor processing techniques are now being used to produce patterned LEDs having multiple micro-emitter elements that can be addressed individually [85]. Commencing in July 2004, the Research Councils UK Basic Technology Research Program "A thousand micro-emitters per square millimetre" focussed on developing micro-structured light emitting diodes having various emitter geometries, sizes, emission wavelengths and electronic drive architectures. Based on processing of III-V nitride semiconductors, the LED devices are specifically designed to suit applications in the fields of microscopy, micro-displays and high-density biochips.

Several considerations are important for the development of flexible and reliable instruments based on the micro-structured LED technology:

- Nitride based semiconductors have been chosen because of their unique physical and light emission properties, particularly in the blue and UV regions.
- The designs of emitter geometries and emitter addressing architectures are driven by specific biological applications. In this area light throughput and timing are critical factors.
- Electrical drivers have been developed to drive the arrays of individually addressable emitters at large currents and high frame rates.

In this chapter basic properties of III-V Nitride semiconductors are described. This family of semiconductor materials enables very bright micro-emitters to be fabricated. A rigorous characterisation of two types of micro-structured LED arrays (a 64x64 point emitter array and a 120-stripe array) is presented together with specific electrical drivers that enable the fast display of arbitrary patterns.

1. Properties of nitride semiconductor devices

III-V Nitride semiconductors such as Aluminium-Nitride (AlN), Gallium-Nitride (GaN), Indium-Nitride (InN) and their alloys emerged in the mid 90's as a very promising material system for LED manufacturing. Their properties make them ideal candidates for the design of high brightness micro-structured LEDs. Nitride compounds can emit light across the entire visible spectrum and into the ultraviolet - wavelengths frequently used in many biology applications. Nitride materials are extremely robust and can be driven at very high current densities. By processing the semiconductor wafers into micro-structured devices, arrays of emitters with different geometries were fabricated, such as matrix arrays of circular emitters and arrays of stripe emitters. For both geometries light emission is achieved either from the top of the diode using the sloped wall mesa emission technology or from the device substrate using the flip-chip emission.

1.1. Band gap structure and emission wavelength

Nitride semiconductor materials and their alloys cover an energy band gap range of 0.7-6.2 eV [86, 87]. Therefore, the entire visible range and deep UV wavelengths are spanned in the nitride alloy system. The band structure is currently thought to be a direct band gap across the entire alloy range. The probability of an electron from the valence band recombining with a hole from the conduction band under appropriate

forward voltage is therefore high since momentum will be conserved upon recombination, allowing nitride-based LEDs to exhibit quantum efficiencies as high as 12% [22], significantly higher than most other semiconductor systems. Table 1 below summarizes the energy band gaps and emission wavelengths of different nitride-based materials.

Material	Energy Band Gap E_g (eV)	Wavelength (nm)
InN	0.7	1700
GaN	3.4	360
AlN	6.2	197

Table 1: Energy band gap and emission wavelength of nitride semiconductor materials

By varying the nature and proportions of the alloy in the semiconductor the nitride material system can cover a wide spectrum, theoretically from 1700 nm down to 197 nm, from infra-red light down to deep UV light according to the relation:

$$E_g = h\nu = \frac{hc}{\lambda} \quad (3.1)$$

where E_g denotes the energy band gap of the material, h is the Planck constant, c is the speed of light and λ is the free space emission wavelength. However, the shorter the emission wavelength into the UV, the more challenging it technically becomes to generate UV light emission. This is mainly due to a less mature fabrication process.

Finally there is also an issue fabricating devices that emit in the green/yellow part of the spectrum between 550 and 620 nm. Because the very large lattice mismatch between InN and GaN, the increasing fraction of InN that is necessary to decrease the bandgap causes a large stress into the material, which results into a very weak emission.

1.2. Threshold voltage

In highly doped semiconductor materials, like the material used for micro-structured LEDs fabrication, the theoretical voltage required to turn on an emitter can be approximated by the band gap energy divided by the elementary charge [88]

$$V_{th} \approx \frac{E_g}{e} \quad (3.2)$$

However, in practice the turn-on voltage of micro-structured elements is often measured to be higher mainly due to voltage drops in emitter contacts.

1.3. Physical properties

Nitride semiconductors possess outstanding physical properties, which make them particularly attractive for reliable applications. These wide band gap materials possess high thermal conductivity (typically $1.3 \text{ W.cm}^{-1}.\text{°C}^{-1}$ for GaN), very high melting temperatures of the order of 2500 to 3000 °C, and high bond strength that make them inherently robust compounds [22]. The high thermal conductivity also makes efficient heat extraction possible, thus increasing the current limit at which thermal saturation commences. In addition, nitride semiconductors are resistant to chemical etching and GaN devices can be operated in harsh environments. Assuming the heat produced inside the LED package is efficiently extracted, nitride micro-LEDs can be driven at very high current density of the order of 20 kA.cm^{-2} [89].

These features make nitride-based LEDs bright, reliable and robust, and manufacturers often claim indefinite device lifetime, usually much more than 100,000-hour operation.

It is worth mentioning that these physical properties are true despite the relatively high dislocation density found in nitride semiconductors [22].

1.4. Spectral radiance

Radiance is the most meaningful quantity when comparing the performance of various incoherent microscopy sources. Radiance is a measure of the light flux per unit solid viewing angle and is expressed in watts per square metre per steradian. This quantity takes into account the size of the emitter and the source emission profile. When an optical system is usually needed to project the excitation light source onto the specimen it fundamentally cannot increase radiance. To increase the density of photons received by the sample, one can only increase the radiance of the source by increasing the number of photons produced, or by increasing the numerical aperture of the projection optics at the sample. In fluorescence microscopy, only a small spectral window is used to excite the fluorescence from the sample so the variation in radiance across the required spectral range should be considered when comparing

traditional microscopy lamps with light emitting diodes, whose emission inherently lies in a narrow spectral range.

Light budget is a critical factor in fluorescence microscopy as usually fluorophore cross-sections and quantum efficiencies are very low. This is why arc lamps, such as Xenon and Mercury lamps, or halogen lamps with high radiance have been traditionally used for this application. The optical power of different light sources used in fluorescence microscopy, including commercially available LEDs, were systematically measured in the specimen plane by Nolte et al [90] for different wavelengths. At the time, the power from nitride LEDs in the sample plane was found to be about one order of magnitude lower than traditional high-pressure arc lamps across the UV and visible spectrum, but the radiance of LEDs is expected to increase rapidly as the LED technology improves. Nonetheless the radiance of high-brightness diodes was shown to be sufficient for many fluorescence applications.

1.5. LED device structure

Two main technologies have been used to fabricate micro-structured LEDs. The first-generation devices have a top emitting structure whereas the second-generation devices were fabricated using the flip-chip technology, which allows for brighter and more uniform emitters. Both top emission and flip-chip LED technologies are based on very similar processing techniques that were developed recently, but light extraction is much more efficient in flip-chipped devices as the light does not have to be emitted through conductive metalisation layers.

1.5.1 *Top emission devices*

The generic device structure for a single nitride emitter having top emission is shown in Fig. 3.1. For simplicity, one can consider that n-doped semiconductor material is grown on a substrate, such as sapphire or GaN, and the active region, where the light emission takes place, is sandwiched between the n-doped material and a thinner layer of p-doped semiconductor material. The thickness of the substrate layer is of the order of a few hundreds of microns, the n-doped material layer is usually a few micron thick, the active region composed of quantum wells is only tens of nanometres thick depending on the number of wells and the p-doped semiconductor layer is hundreds of nanometre thick. The device structure itself is only a few microns in thickness and a

total of less than half a millimetre in thickness including the substrate. The composition of the semiconductor alloys used in both the n-doped and the p-doped semiconductor materials, together with the quantum well thickness determine the band gap structure and thus the emission wavelength.

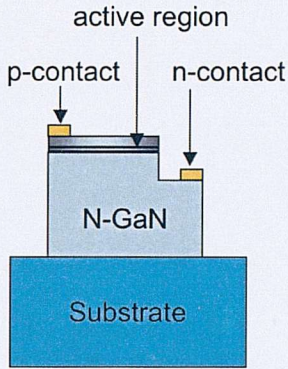


Fig. 3.1. Generic top emitting LED structure

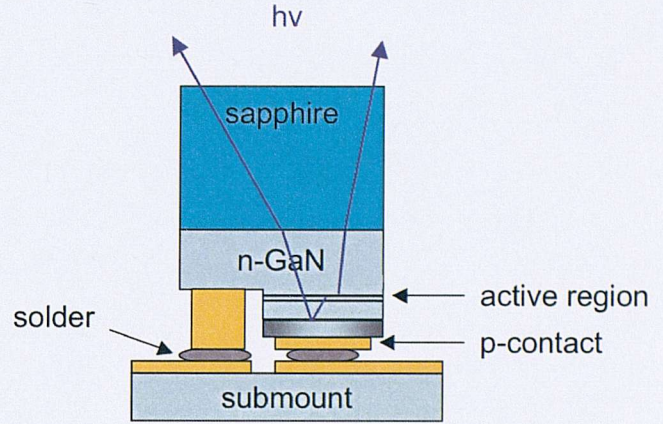


Fig. 3.2. Flip-chip LED structure

When a positive potential difference is applied across the diode (*i.e.* the diode is forward biased), carriers are injected in the quantum wells where they radiatively combine and light emission occurs from the top, sides and bottom of the device. Standard nitride semiconductor wafers can be readily purchased, emitting at different wavelengths from deep UV to green. Full descriptions regarding the semiconductor material and processing can be found in Jeon et al. [23, 24]

1.5.2 Flip-chip LEDs

Flip chip technology has been recently developed for nitride high power LED fabrication [91-93]. In this technology the LED is flipped over or inverted compared to a conventional top-emission device and light extraction is achieved through the transparent substrate as shown in Fig. 3.2.

Flip-chip LEDs have advantages over conventional devices in terms of light extraction, due to light reflecting from the metal p-contact back to the sapphire substrate and because it no longer needs to pass through the semitransparent metallic current spreading layer that is often used on top emission devices. In addition special light extraction techniques can be applied to avoid total internal reflection at the

GaN-sapphire and sapphire-air interfaces due to the relatively large refractive index difference [94-97].

The flip-chip technology has advantages in terms of heat extraction [98], and hence much higher driving current densities can be used. As the diode is bump soldered on large n and p contacts, resistance is also reduced. Together with placing an electrical driver near the diode contacts for lower capacitance, this allows to pulse flip-chip LEDs in the sub-nanosecond regime for time-resolved spectroscopy measurements [99].

Finally, CMOS electronics can be integrated on the diode sub-mount for compact and versatile electrical driving. This is particularly attractive when trying to address large matrix arrays of flip-chipped emitters, as CMOS technology enables them individually addressed with no need for an increasing number of on chip connections.

2. Matrix arrays

The first micro-emitter matrix LEDs that were developed were top-emitting 12µm diameter GaN micro-disk LEDs [100] emitting at a wavelength of 408nm. Further developments came by connecting hundreds of 9 and 12µm diameter emitters together on the same 300x300µm wafer [101]. This resulted in an increased optical flux compared to broad area LEDs. These developments eventually led to the first 10x10 pixel nitride micro-display emitting at 450nm [85]. Each element in the display was connected to individual n and p terminals, which resulted in 200 connections. This direct addressing scheme proved possible to display simple fixed patterns such as single characters without multiplexing.

Other groups started to concentrate on a matrix interconnect scheme to drive top emission LED arrays in a realistic way. Using a matrix interconnect driving scheme a single row electrode is activated at a time while a pattern is displayed on the column electrodes. The row pattern is then time-multiplexed (raster scanned) to produce a 2-D image. A direct addressing scheme requires up to $2N^2$ connections to drive a $N \times N$ element array whereas addressing the same array with a time multiplexed matrix scheme only requires $2N$ connections. Arrays of much bigger scale can then be designed. Matrix-addressable GaN array of 32x32 elements were demonstrated by running electrical contacts along its columns (cathodes) and rows (anodes) [102].

Instead of being addressed individually, each element could be turned on by applying a voltage difference on the corresponding row and column.

Shortly afterwards, high-density LED arrays where each element was matrix-addressed were designed and fabricated by the Institute of Photonics at Strathclyde University. Over the course of the Basic Technology Research Programme the processes and designs of the LEDs has been refined with particular emphasis on uniformity and increased radiance.

2.1. First generation micro-displays

A summary of the steps required to process a semiconductor wafer into an LED matrix device is presented below and schematically in Fig. 3.3. Greater detail can be found in reference [25]:

- A periodic rectangular mesa structure is etched first (Fig. 3.3a). This defines the columns. All the emitters in a column share a common n-electrode by connecting the mesa structure to a single pad.
- The Mesa structures are isolated from each other by filling the gaps with a non-conductive material such as SiO_2 (Fig. 3.3b).
- Planarization for height uniformity is carried out to facilitate the deposition of p-electrodes.
- Semi-transparent metal Ni-Au p-electrodes are deposited across the mesa structure, which results in connecting all the emitters from a row together (Fig. 3.3c).

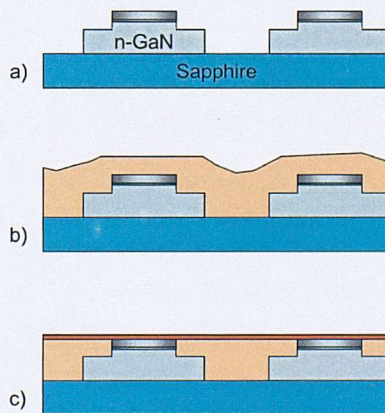


Fig. 3.3. First generation matrix processing

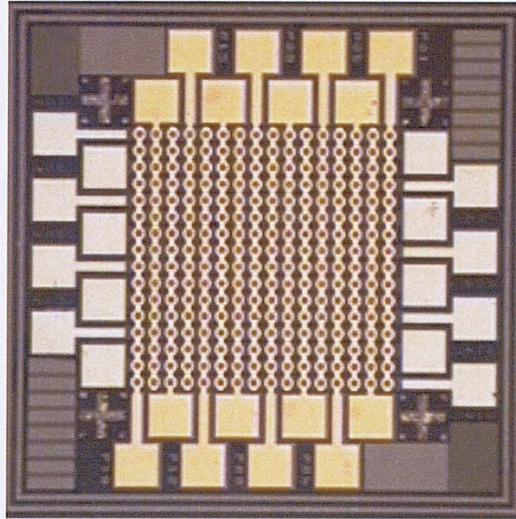


Fig. 3.4. Microscope image of a 16x16 LED array

Fig. 3.4 shows a top-image of a 16x16 LED matrix. 16x16, 64x64 [23] and 128x96 [25] nitride LED matrices have been reported emitting in the green, blue and UV [26]. Other groups have also reported recently the fabrication of 10x10 UV LED array using a similar wafer processing technique [103]. A new tapered sidewall etching technique was also developed [104, 105] that allows for easier p-electrode deposition and better optical performance.

In the first generation design, 16 μ m diameter aperture emitters are separated by 30 μ m from centre to centre. As seen on Fig. 3.4, for each column and row, contacts are made on one side of the array and electrodes run the entire length of the device structure to connect the emitters. The connection side is alternated for a more compact pad arrangement. In Fig. 3.4, yellow connections running on top of the mesa structure (top to bottom) are anodes and white connections running underneath (right to left) are mesa cathodes. When turning on the first blue 64x64 array at constant 3.2V as shown in Fig. 3.5, several observations can be made:

- Ultra-bright emitters are randomly distributed across the matrix.
- Emitters gradually become dimmer away from the cathode bond pad (Fig. 3.6), which suggests a significant voltage drop along the n-GaN mesas. Some pixels at the end of the columns are not even turned on under 4.2V forward voltage.
- Brightness remains unchanged along a single column of emitters, which suggests little voltage variations along the metallic p-electrode.

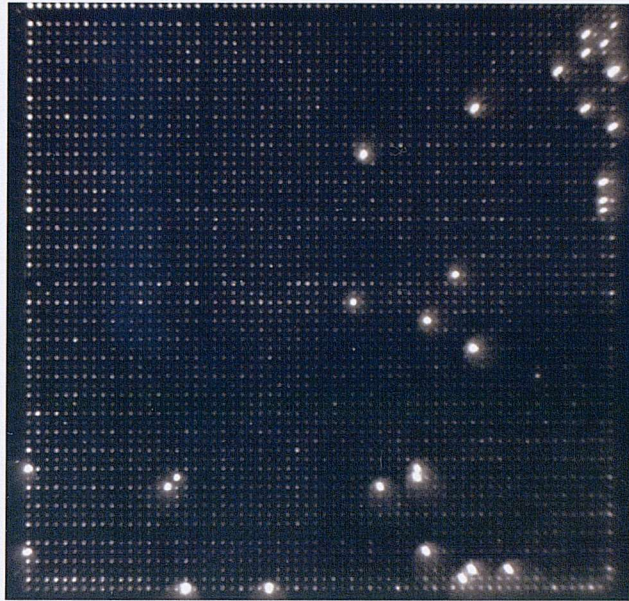


Fig. 3.5. 4x microscope image of a blue 64x64 array driven at 3.2V



Fig. 3.6. 10x microscope image of three rows of emitters turned on at 3.2V.



Fig. 3.7. 10x microscope image of a column of emitters turned on at 3.2V.

Ultra-bright defects are thought to be due to non-uniform p-metal deposition. A better connection results in a lower resistance at the emitter, which then results in a brighter emission when driving the whole array at a given voltage.

The decrease in output power away from the bond pad can be explained by the series resistance increasing linearly along the mesas. The matrix structure is presented schematically in Fig. 3.8, where the resistance of the p-electrode is not shown. By analysing the I-V curve of the corresponding LED pixels in a column, the resistance was measured to increase from $2.4\text{k}\Omega$ to $10.7\text{k}\Omega$, showing nearly 5-fold increase in resistance from the start to the end of the n-mesa. The resistance of the p-

line was measured to be around $1.9\text{k}\Omega$ with little variation along the p-electrode, as illustrated in Fig. 3.7. The turn on voltage was also found to vary from 2.7V to 4.5V.

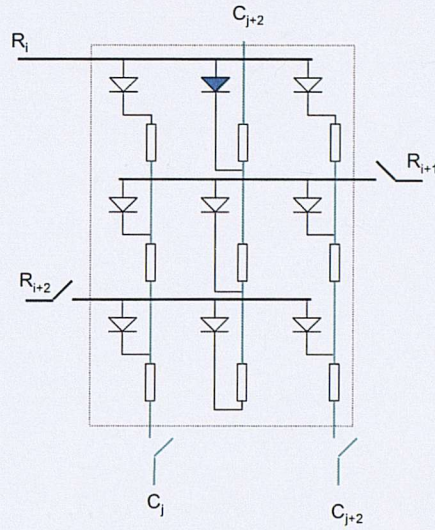


Fig. 3.8. Schematics of a matrix device portion

The following three conclusions can be made:

- Emitters in the device should be driven at constant current rather than constant voltage when designing an electrical driver. This is because, in general, the intensity of emission is directly proportional to the current through the diode and so the LED driver should be using constant current sources.
- A new device structure lowering and homogenising the resistance along the rows should help reduce the operating voltage and give higher brightness and better uniformity. A new device structure was then designed and is reported in the next section.

2.2. Second generation device: improving emission uniformity

In order to tackle emission non-uniformities and large series resistances, the main causes of these non-uniformities were systemically investigated. A simple analytical model was established [28] that helped the optimization of the device structure. A new process to improve the device performance was developed, which includes:

- A $4\mu\text{m}$ wide side-rail metal electrode, which is introduced to suppress the resistance variation along the n-mesa and thus improves the emission uniformity across the LED matrix.

- A new spreading layer formation scheme to decrease the turn-on voltage and increase light output of each pixel, as it can be driven harder under the same forward voltage.
- An additional ring-shaped contact on top of the spreading layer to act as a sacrificial layer during the plasma etching process so that the thin spreading layer does not experience plasma damage.

Fig. 3.9 shows a structural diagram of a new device. Because of the n-metal line, the pitch size had to be increased from $30\mu\text{m}$ to $50\mu\text{m}$, with the same $16\mu\text{m}$ emitter diameter.

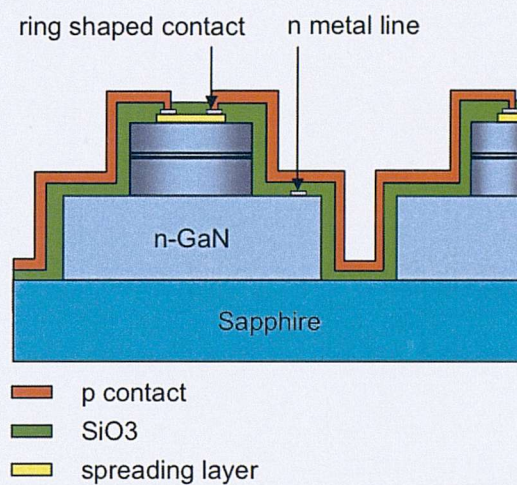


Fig. 3.9 Cross section of the improved emission matrix device.

The geometrical and electrical characteristics of a conventional and improved blue matrix devices are summarized in Table 2. The resistance for each pixel of a blue matrix device was measured to vary from 560Ω to 601Ω from the beginning to the end of the mesa structure. These results indicate that the metal line can efficiently reduce the resistance variation along the n-GaN length down to 8% compared to a factor 5 for the previous design, from 2.4 to $10.7\text{ k}\Omega$. The additional ring-shaped p-contact on top of the emitters resulted in a lower resistance of the p-connections. This improved contact explains why the total series resistance of the improved device is even lower than the p-resistance alone of the first generation device.

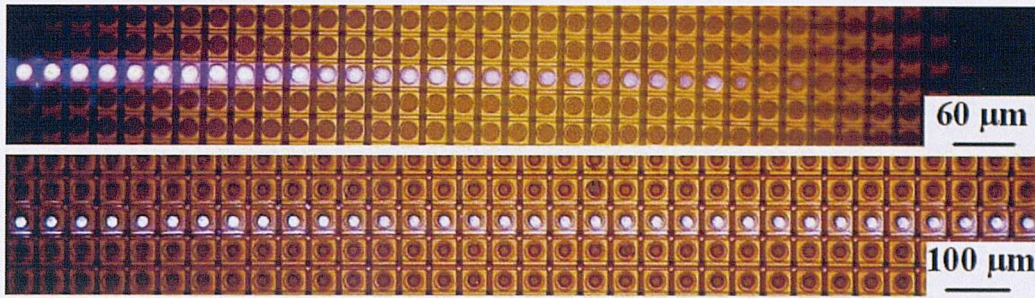


Fig. 3.10 Emission uniformity in a row of pixels in a first-generation (top) and a second-generation matrix device driven at a constant voltage.

Because the lower series resistance, emitters can be driven at much higher current, up to 10 mA per emitter for the blue device before breakdown.

2.3. Third generation matrix: improving heat extraction and external quantum efficiency.

In the second-generation top-emitting devices, the power-output for individual pixels is still relatively small and is mainly limited by the small emission area and low external quantum efficiency. Indeed much of the light is still either absorbed by the semi-transparent metal electrode or trapped inside the diode by multiple reflections. The current handling is also poor, mainly due to the contact resistance and temperature rise at high current. All these issues can be addressed by using the flip-chip technology described in the first section.

A new device with 64x64 pixels was fabricated, maintaining the same rectangular mesa structure and interconnect addressing scheme used for the second-generation top-emitting device. Each of the pixels has a 20μm diameter emission aperture on a 50μm pitch. Instead of the thin Ni/Au spreading layer, a thick Ni/Au/Ti/Au multilayer is grown by evaporation as reflective p-contacts. This guarantees the formation of low contact resistance and highly reflective p-contact. The ring-shaped contact was no longer necessary in the flip-chip design. After the device fabrication, the substrate of the chip is polished by chemical mechanical polishing (CMP) and the device is bump-soldered on a silicon mount having Ti/Au metal tracks. The tracks are connected to pads such that the whole matrix can be addressed using the same multiplexed matrix interconnect scheme.

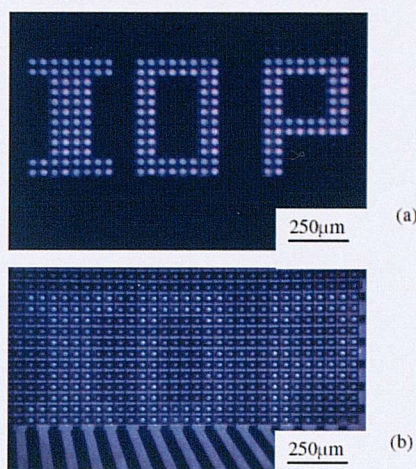


Fig. 3.11. Representative micro-display programmed onto (a) the flip chip blue micro-LED array and (b) the top-emitting second generation blue micro-LED array

Fig. 3.11 shows the same pattern displayed with both the flip-chipped and top-emitting devices. Good uniformity and low light scattering is demonstrated for the flip-chipped LED. This constitutes an improvement over the top-emission devices shown in Fig. 3.11 (b), where the brightness and contrast is poorer under the same driving conditions. Light scattering for top-emission device is sufficiently strong that the non-emitting area is also lighting up, which results in reduced contrast.

2.4. Characterization of micro-LED arrays

2.4.1 *Emission spectra*

The emission spectra of the blue and UV arrays are shown in Fig. 3.12. Emitters were driven at 3mA per emitter and spectra were recorded with an Ocean Optics spectrometer. Blue emission is centred on 465nm with a FWHM of 22nm and UV emission is centred on 371nm with a FWHM of 11nm. A longer spectral tail is always present in the blue emission, due to the semi-conductor composition. Because the emission wavelength depends mainly on the semi-conductor material, it should be the same whatever the emitter geometry. However, when driver at very high current densities, the emission spectrum can broaden slightly and angular dependences have also been reported elsewhere [106]. An appropriate filter can be used to clean up the emission spectrum when required.

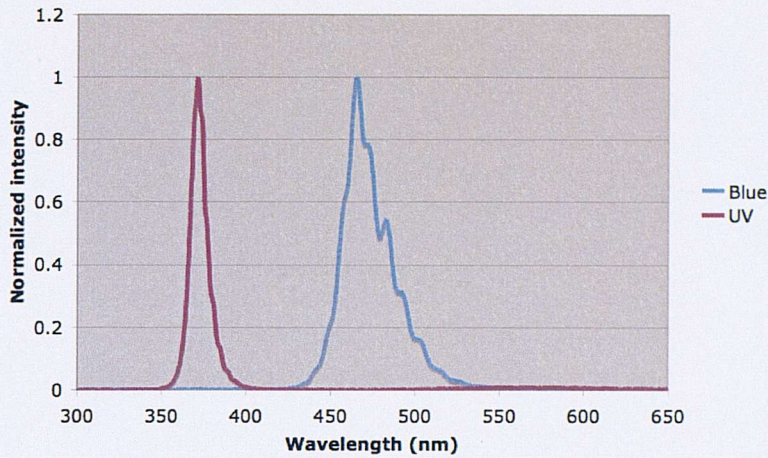


Fig. 3.12. UV and blue emission spectra.

2.4.2 Electrical characteristics

Emissions from 64x64 devices emitting at 368nm and 470nm were characterized for all three generations of devices. For the first generation devices, emitters located at the beginning of a row were chosen in order to avoid voltage drops due to the increasing resistance along the mesa.

I-V curves

Fig. 3.13. and Fig. 3.14 show the typical current-voltage (I-V) characteristics of an individual pixel in the flip-chip and top-emitting arrays, emitting at 470nm and 370nm. Turn-on voltages were measured to be 2.7V and 3.6V for blue and UV devices, slightly higher than predicted by the band gap energy of 2.6V and 3.32V, respectively.

At the same emission wavelength, the I-V characteristic of flip-chip device is marginally steeper after turn-on than the top-emission one. This suggests that the series resistance of the emitter p-contact is lower in the flip-chip case. Given that the p-contact is 20 μ m diameter for the flip-chip device instead of 16 μ m diameter for the top emission one and considering that the p-contact of the flip-chip device is made from a higher conductivity material, this resistance improvement is understandable.

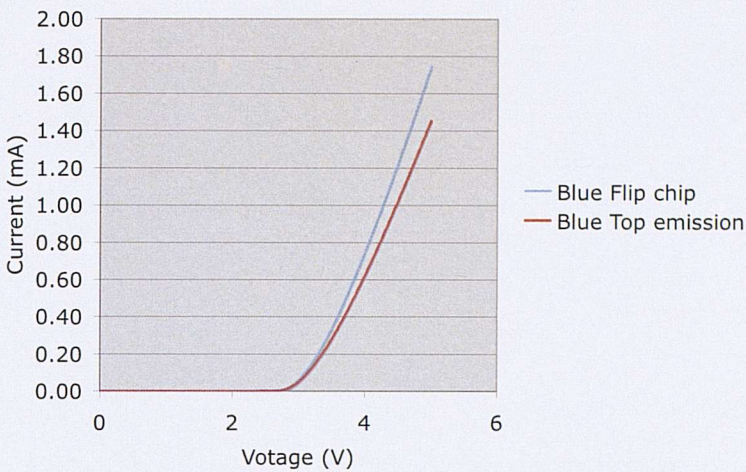


Fig. 3.13 I-V curves of a single blue emitter: flip chip (blue) and top emission (red) LED

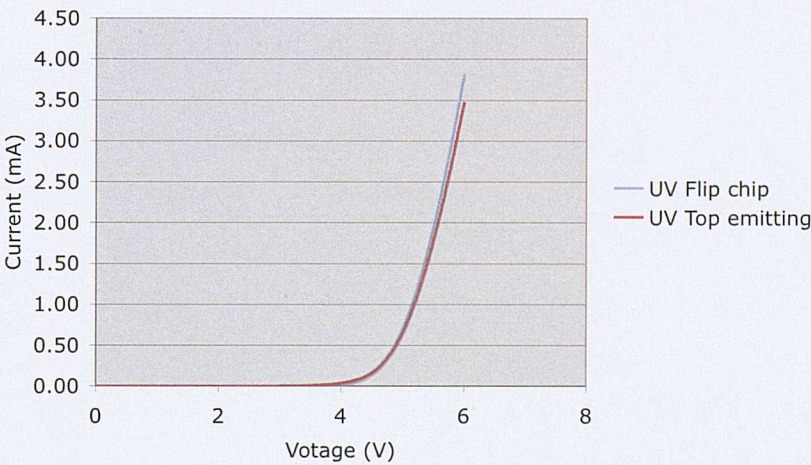


Fig. 3.14. I-V curves of a single UV emitter: flip chip (blue) and top emission (red) LED

Optical power

Optical output powers from single emitters are measured using a Newport 1918-C power-meter. In order to make accurate comparison of the light emission capability for different device geometries, irradiances of single blue emitters are calculated and shown in Fig. 3.15. Clearly, under the same injection current density, the flip-chip device can provide a much larger light output power density than the top emission ones. For instance, at a current density of $500\text{A}/\text{cm}^2$, a power density of $10\text{ W}/\text{cm}^2$ can be achieved from the flip-chip blue LED, twice as large as that for the second generation top-emitting one. The power enhancement of flip-chip InGaN broad area devices is well documented [107, 108]. First, the light in flip-chip LEDs emits through the transparent sapphire substrate instead of the semi-transparent electrode. Hence

much more light can be extracted due to the reduced light absorption. Second, with a thick, reflective p-contact, the current spreading is considered to be more uniform within the emission area. The same arguments are valid for the flip chip micro-LED arrays here.

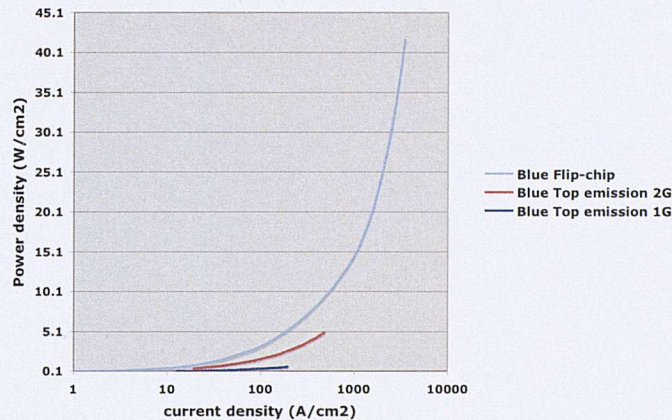


Fig. 3.15. Power density versus current density for the Flip chip (light blue), and top emission first (red) and second generation (dark blue) blue 64x64 emitter arrays..

It should be noted that flip-chip devices can sustain an extremely high current density up to 4000 A/cm² before breaking down, reaching a maximum power density of 43 W/cm². By contrast, the maximum power density before failure of an emitter in the top LED blue array is 5 W/cm². This performance enhancement is attributed to the improved thermal dissipation after the silicon mount is introduced and further improvements are expected when introducing an AlN mount [103].

The same measurements are taken for the UV devices and shown in Fig. 3.16. At 500 A/cm², the flip-chip device is 1.5 times brighter than the top emission ones. At very high current density, thermal saturation is also observed. A maximum power density of 7 and 3.5 W/cm² was measured before breakdown for the flip chip and top emission array, respectively.

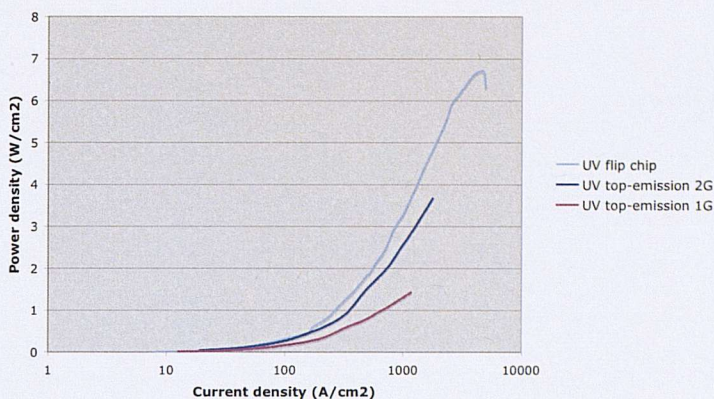


Fig. 3.16. Power densities for the flip chipped (light blue), second generation (dark blue) and first generation (purple) UV matrix devices.

2.4.3 Device comparisons

Table 2 summarizes the main characteristics of the blue and UV matrix devices. UV characteristics are quoted inside brackets. During the course of the Basic Technology research program the technology has been clearly improved in terms of resistance uniformity, heat management and sustainable output power.

	1G top	2G top	Flip-chip
Pitch size (μm)	30	50	50
Emitter diameter (μm)	16	16	20
Series resistance (kΩ)	2.4-10.7	0.56-0.60	0.4-0.5
Maximum current per emitter (mA)	2 (9)	10 (14)	50 (62)
Maximum output power for a single emitter (μW)	5 (11)	40 (24)	524 (79)
Number of pixels	64 x 64	64 x 64	64 x 64
Device size (mm²)	3.5	9.9	9.9

Table 2. Geometrical and electrical characteristics for blue (and UV) matrix devices

2.5. Driver for multiplexed display

As discussed in section 2.1, driving all the emitters in parallel by applying a constant voltage across the array is often not optimal for LED matrix devices as the resistance non-uniformities induce large differences in the emission output as illustrated in Fig. 3.5. Hence a specific driver able to compensate for those resistance variations is required, driving the emitter at constant current instead of constant voltage in order to obtain a reliable light output. Such a driver was designed and fabricated. It relies on programmable voltage sources to drive the anodes and programmable constant current sinks on the cathode side to drive a constant current through each emitter.

2.5.1 *Strategy to drive arrays of individual emitters*

Multiplexing

Multiplexing is a common technique employed to operate LED matrices. By multiplexing, only one row – or cathode – of the LED matrix is activated at any one time. This approach is required because connecting an anode activates the whole row of emitters. Fig. 3.17 shows the case when a voltage is applied to both rows A and B at the same time. It becomes impossible to address an individual LED within those two rows. Hence, only one row should be activated at a time and an LED pattern should be displayed using the constant current sources on the column side. Raster-scanning the rows in quick succession and changing the corresponding column pattern will then allow the display of any arbitrary binary pattern or image.

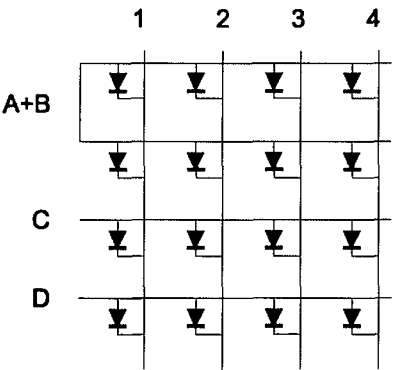


Fig. 3.17 LED array schematics. Because the anodes A and B are connected together, any column will turn on two LEDs at the same time.

Brightness control

The brightness of an LED is roughly proportional to the current that flows through it. Changing the current value then changes the light output. However, this is not recommended as this implies very accurate constant current sources/sinks for which the current level can be rapidly tuned. This is in practice difficult to achieve. A more reliable technique for brightness control is through pulse width modulation (PWM), where the LED is flashed periodically. The brightness can be finely tuned by changing the duty cycle (ratio of the on period over the flashing period), as illustrated in Fig. 3.18. The switching time of an LED being of the order of tens of nanoseconds, a PWM frequency ($1/T_p$) of several kilohertz to megahertz can be used without the switching time becoming noticeable.

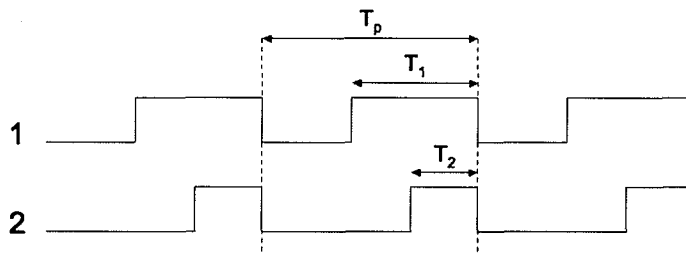


Fig. 3.18 PWM principles. The LED on-time T_2 is half T_1 . Thus LED2 will appear twice less bright as LED1.

Driver structure for interconnected arrays of emitters

As illustrated in Fig. 3.19, the main blocks of the LED array driver are:

- The programmable common-line drivers: They supply a given voltage to the rows activated by the shift register and allow for raster scanning the rows if only a single row is activated at a time.
- The constant current sinks: Assuming that the voltage applied at the diode anode is large enough, a constant-current sink applies an appropriate voltage at the cathode such that the current flowing through the emitter is fixed. This allows for precise current to be driven. The current value can be modified by setting the value of an external resistor. Constant current drivers are usually fast enough to be pulse-width-modulated.
- The driver controller: An external controller handles the communication with the user, stores the images to be displayed, generates the patterns to be sent to the shift registers of the voltage and current sources and looks after the timing conditions such as the raster scanning frequency and the PWM.

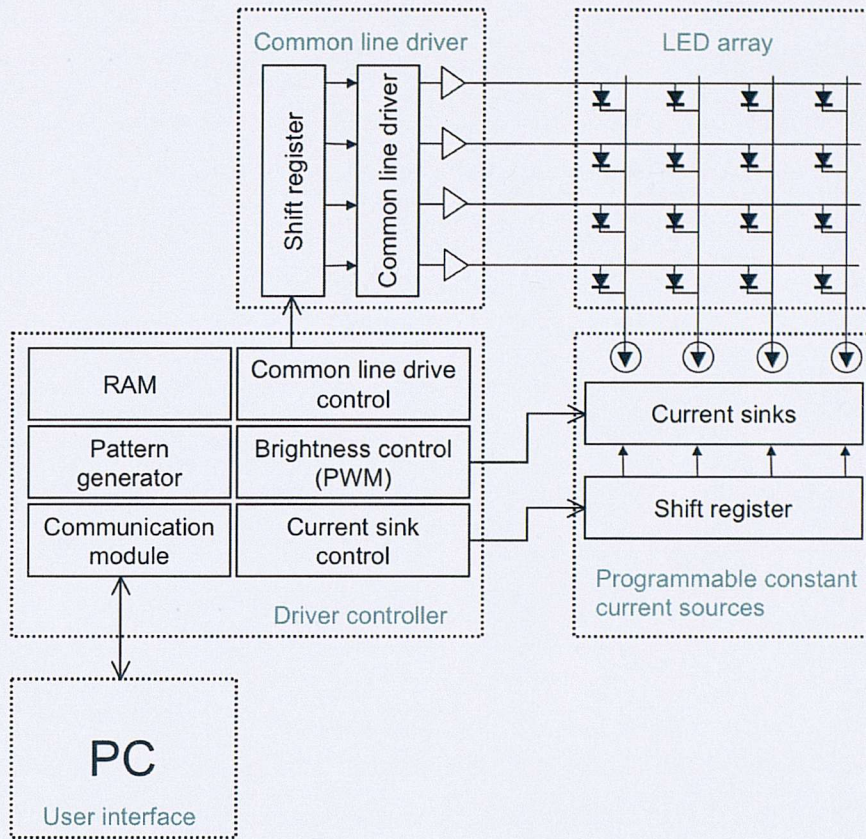


Fig. 3.19. Structure of the LED matrix driver

When displaying an image, the whole array should be raster-scanned sufficiently fast that the observer does not perceive any discontinuity between two consecutive frames. For a human eye frame rates of at least 50 frames per second are sufficient, which is very slow compared to the capability of most common line drivers.

2.5.2 *Matrix driver hardware*

Based on these principles, an electrical driver for 64x64 emitter arrays was designed and fabricated (Fig. 3.20). The mains components are the following:

Microprocessor

The driver board is controlled by a PIC18F4550 microcontroller working at 48MHz. It handles the communications with the host PC through an integrated USB port and it stores the transferred binary frames in the internal RAM memory. A dedicated pin controls the data sent to the common-line registers thus the raster scanning. Data are sent to the current chips using a Serial Port Interface (SPI) enabling rapid modification of the pattern to be displayed at the columns on a microsecond timescale. The

processor looks after the time dependant processes, such as the PWM to control the emitter brightness, the raster scanning speed and the total amount of time the array is turned on. Also, it generates appropriate trigger signals for synchronisation of camera acquisitions and subsequent measurements. The processor is programmed via a dedicated port and can be reprogrammed and debugged at anytime.

Common line drivers

Rows are energized using eight MIC5891N chips. Each of these high-voltage, high-current latched drivers has 8 open emitter outputs able to source up to 500mA and sustaining at least 35V in the on-state. Power is supplied through BNC port connected to an external power supply. The chips are serially connected together so that the shift registers can be addressed using a single controller pin.

Constant current sinks

Constant currents are sunk on the column side using four MAX6971AUG chips. Each chip has 16 constant current outputs rated at 36V, which are able to sink from 3mA to 55mA per output. These chips are connected in series. A dedicated output-enable pin gates all 16 outputs in synchrony and allows PWM for intensity control. To electronically modify the constant current value, a quad 256-step digital potentiometer controlled by the board microcontroller sets the external resistor values. The microprocessor writes the potentiometer resistance values using the fast SPI port. The SPI is shared between the potentiometer and the current sources, so an extra multiplexer is added that allows for chip selection.

Trigger lines

Two triggers lines were included (parallel BNC ports in Fig. 3.20) to synchronize the illumination with other instruments, such as CCD camera or a micro-electrode array.

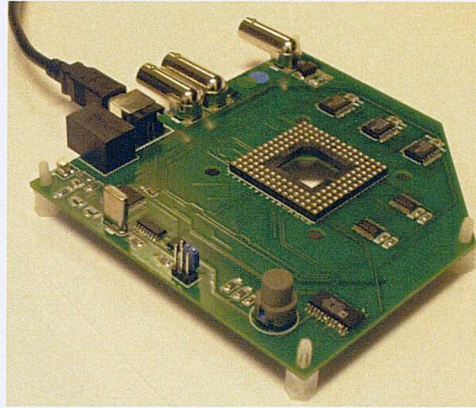


Fig. 3.20. Electrical driver for 64x64 LED matrix.

2.5.3 Microprocessor firmware

USB communication

A virtual RS-232 serial port is emulated in the board processor on top of the USB port such that the host computer detects a conventional RS-232 port when the driver board is connected to its USB socket. Data transfer speed is however much faster than for a conventional serial port and is only limited by the capability of the integrated Full-Speed USB port, i.e. 12Mbits/s. Such a virtual port makes it easier to design a user interface as the existing RS-232 communication routines can be readily used.

After the processor initialization, data sent from the host computer to the driver board are automatically stored into the USB buffer. Strings of up to 64 bytes (or characters) can be transferred at once before emptying the buffer and a state-machine is implemented that continuously polls whether data have been sent (Fig. 3.21). Accordingly, retrieved data are stored in on-chip RAM memory.

Display modes

Several display modes are implemented. Two display modes use raster scanning, namely the Image and Scan_Square modes, whereas two modes use continuous illumination, the Square and Single_Pixel modes.

In Image mode all the 64 rows are rapidly scanned one after another. For each row the corresponding pattern is displayed on the column side. When raster scanning the full array, the average brightness of the emitters is reduced by a factor 64 compared to constant illumination because each emitter is turned on for $1/64^{\text{th}}$ of the time. This driver can display up to 450 binary images per second when PWM is inactive and is limited by the processor speed.

In Scan_Square mode, a rectangular area of the array is selected by sending the (left, right, bottom, top) coordinates to the board. Raster scanning only occurs on the rows of interest, which makes the frame rate faster and the emitters brighter.

In the continuous illumination modes, all the selected emitters are turned on at the same time. These modes enable much larger light output from the LED array, but at the expense of non-uniformities due to emitter-to-emitter variations in the contact resistances. In Square mode, the coordinates defining a square area are sent to the board and the area is constantly turned on. In Single_Pixel mode, only a single selected pixel is turned on at any time.

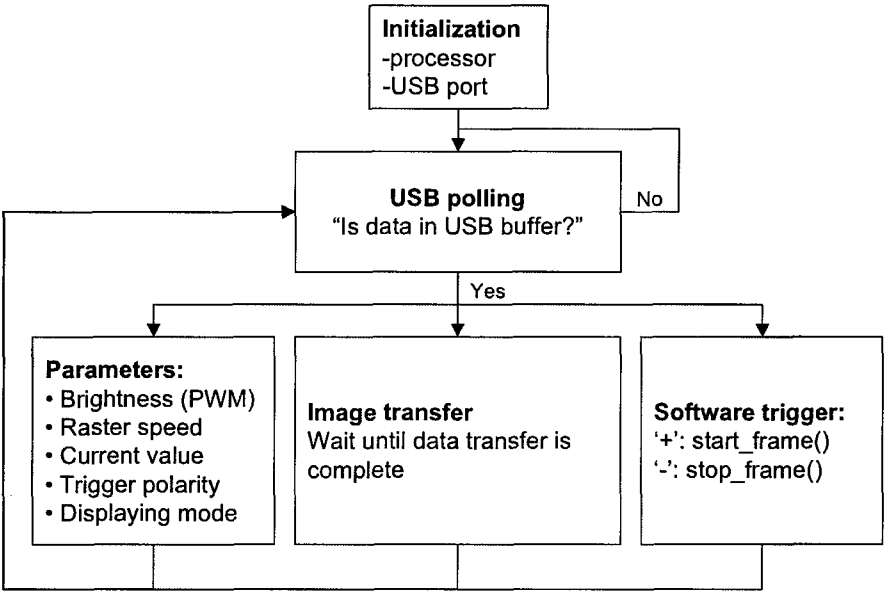


Fig. 3.21. Processor state-machine flowchart

The array is turned on when the processor receives a 1-byte ('+') software trigger, which enables interrupts and starts timer counters. Timer3 is dedicated to raster scanning (from 40 μ s to 5 ms per row) and Timer0 sets the time the array remains on before being automatically turned off (from ms to 1.5 s). This pulsed mode can be inactivated such that the array remains on all the time. After starting a frame, the processor goes back to USB polling until the timer overflows. The code is then branched to the interrupt routine where the appropriate action, either raster-scanning or turning off the array, is taken.

An extra Pulse_Train mode was included where the driver automatically generates a train of pulses. The pulse length, inter-pulse duration and number of

pulses are fully controllable. This mode was implemented for investigating dynamics of photosensitive neurons, as described in chapter 7.

2.5.4 *User interface*

A Labview interface was designed for user-friendly control of the LED matrix (Fig. 3.22). PWM, scanning speed, constant current values, trigger mode, integration time, pulse train mode and display mode can all be changed quickly. In Image mode, a 64x64 black and white icon is selected by the user to be uploaded into the driver memory.

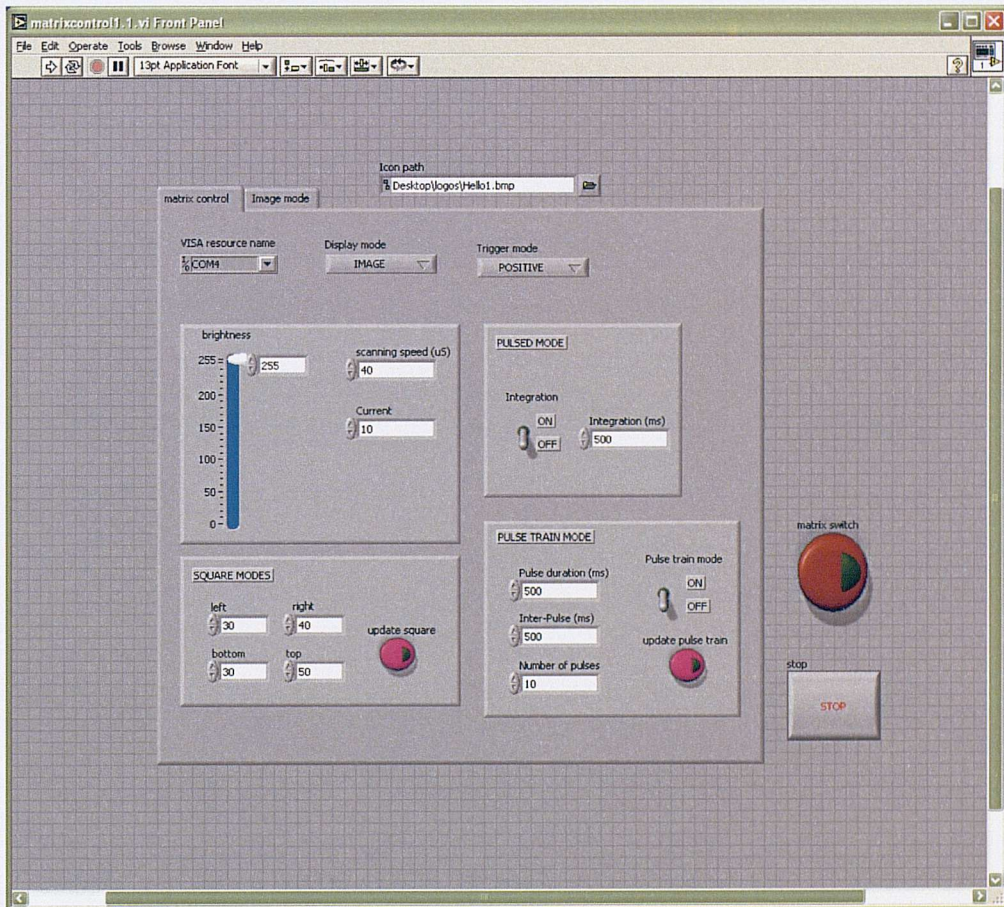


Fig. 3.22. Labview user interface for controlling 64x64 LED arrays

Because the whole system relies on simple string commands, it would be easy to develop more interfaces based on other programming languages, such as C, C++, Matlab, etc. Communication with the driver board would take place through existing RS-232 serial port routines. Fig. 3.23 shows a 64x64 binary icon and the corresponding LED display under 4.5V forward voltage. The image was captured using a CCD camera triggered directly by the driver board. Integration time was 1ms

and scanning speed was $40\mu\text{s}$ per row. A first generation blue LED array is used here. As expected, driving the LEDs at constant current allows for brightness correction compared with Fig. 3.5.

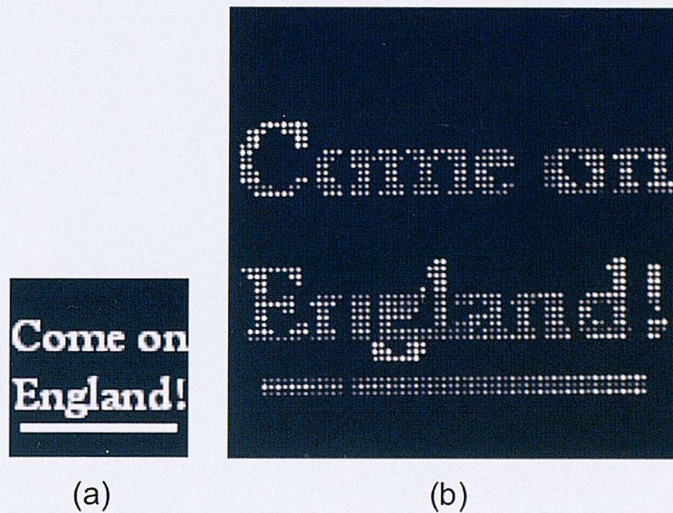


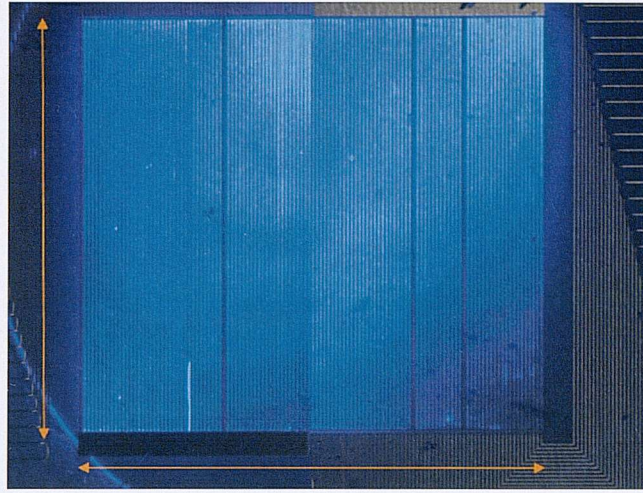
Fig. 3.23. Pattern displayed in Image mode for the Football World Cup 2006: (a) 64x64 icon loaded in Labview and (b) 1.25X microscope image of the LED array

3. Micro-stripe LED: performance and driver

3.1. Stripe array geometry

Because the top emission and flip-chip structures are generic, micro-structured devices with different emitter geometries can be fabricated. In order to investigate line scanning microscopy techniques using LED arrays, a top emission microstripe emitter array was first designed [27]. The array is composed of 120 stripes sitting side by side on the same wafer. Each stripe of the device is 17 microns wide and 3600 microns long, with a centre-to-centre spacing between stripes of 34 microns, giving an overall diode structure size of 3.6×4.08 mm (Fig. 3.24). A blue device was predominantly used to demonstrate line-scanning microscopy techniques but green and UV micro-stripe devices have also been fabricated, depending on the semiconductor wafer used. Each individual micro-stripe has an n-electrode rail alongside a light-emitting mesa running to a single common broad area n-electrode contact and each mesa has an individual p-electrode running along its length. A particular stripe is then addressed by sourcing a constant current to its corresponding p-contact with the common cathode held at ground.

3.6 mm



4.08 mm

Fig. 3.24. Picture of a blue microstripe LED

3.2. Performances of microstripe LEDs

3.2.1 *Electrical properties*

Curves of power outputs versus current density for the blue and UV top emitting stripe devices are shown in Fig. 3.25. The output from a stripe shaped emitter is about 5x lower than for a dot emitter. This is believed to be due to the superior heat extraction capability of the dot geometry. However stripe power densities are still higher than common commercially available broad area LEDs [27], which suggest patterning of the semi-conductor may increase their output power.

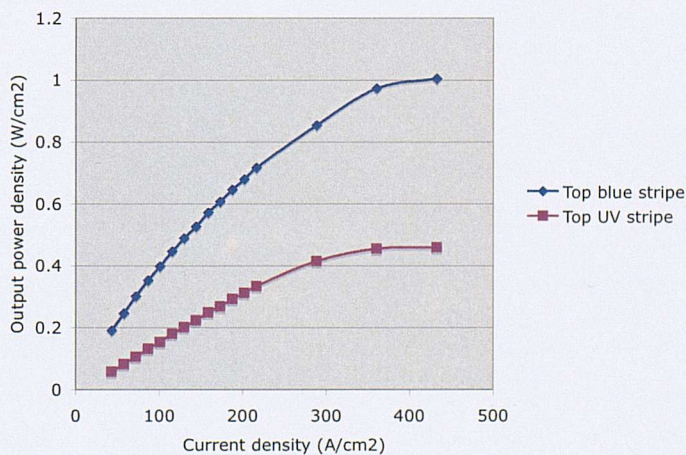


Fig. 3.25. Output power densities for a single blue and UV stripe emitter.

3.2.2 *Emission profile*

The emission profile non-uniformities due to the series resistance of the p electrode increasing along the emitter are shown in Fig. 3.26 for a UV stripe array. Another connection scheme was subsequently designed and a new device is fabricated (Fig. 3.26 (b)) where the p electrode is connected on both ends of the stripe emitter. This connection scheme slightly improves the current spreading. However, the intensity in the middle of the stripe is still three times less bright than at the edges (Fig. 3.27). Flip-chip stripe-LEDs are currently under development where the p semi-transparent electrodes will be replaced by thick metal stripe electrodes. Emission is expected to be much more uniform due to a lower resistance variation along the stripe emitter.

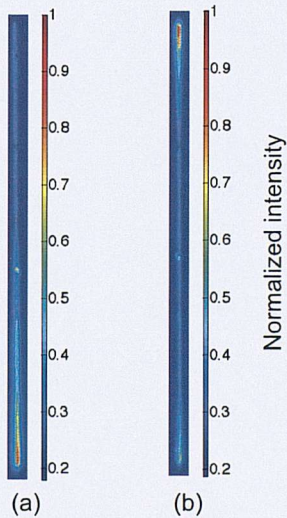


Fig. 3.26. Emission profiles of top emitting stripe UV LEDs (a) without and (b) both sides connections

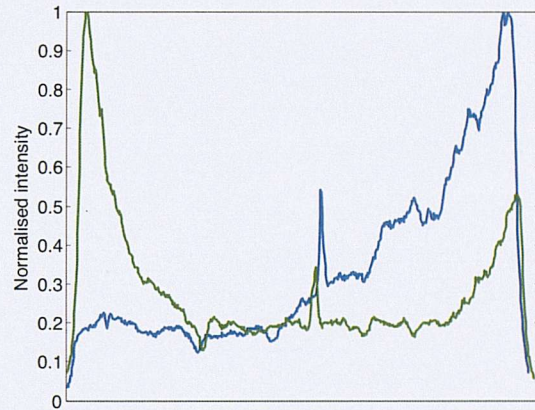


Fig. 3.27. Graphs of the emission profiles

3.3. **Driver for top emission microstripe device**

A dedicated electrical driver was constructed to allow arbitrary combinations of the stripes to be driven simultaneously to produce programmable line patterns. Constant currents are produced using 15 common cathode TB62710F drivers controlled by a PIC18F4550 microcontroller. The communication scheme and device structure are similar with the matrix array driver.

The whole system allowed complete reconfiguration of line patterns in 20 microseconds. The driver board, addressed from a host computer using a LabView™ interface communicating over a USB connection, was designed to control the board parameters and displayed patterns. The constant current value is set using external digital potentiometers on each driver chip, allowing current values from 3 to 90 mA and is reconfigurable in 1.5 μ s. A further 10-bit PWM modulation enables a precise brightness control of the emitters. Overall, the electrical driver is able to display up to 50,000 independent line patterns per second.

4. Summary

A description of the micro-structured LED technology was provided. Arrays of 64x64 point and 120 stripe emitters were fully characterized and diode design was continuously improved throughout the research program. For both types of diode arrays an electrical driver was designed, fabricated, and programmed. Using Labview graphical interfaces allows for rapid control of the pattern being displayed on the diodes up to 450 and 50,000 arbitrary patterns per second for the point array and stripe array, respectively. For each emitter injected current and brightness are controllable in microseconds. These systems will be used in the later chapters describing the applications of micro-LED technology, both in optically sectioned microscopy and stimulation of photo-sensitized neuron cells.

Chapter 4 Grid-projection structured light fluorescence microscopy

If optical sectioning in a microscope is defined as the requirement that all the spatial frequencies, including the zero spatial frequency, attenuate with defocus, it is clear from chapter 1 that a conventional widefield microscope does not have the ability to produce images of the solely in-focus object. Grid projection structured illumination has been introduced as a simple and light efficient widefield technique to encode the imaging plane of interest in order to fill the so-called missing cone. Grid structured illumination has become an established technique for fast and affordable acquisition of confocal images [109, 110].

In this chapter, the basic concept of image formation in a grid-projection structured illumination microscope operating in fluorescence is introduced, which allows for optical sectioning to be achieved with minor modifications of a widefield fluorescence microscope system. The performance of such a system in the presence of photon noise is analysed. When a three-phase process is used to achieve optical sectioning, theory and simulation show that the noise is sinusoidally modulated across the field of view, which needs to be taken into account when making quantitative measurements. Using a blue microstripe LED device, a grid projection structured illumination microscope with no moving parts is demonstrated, where the grid pattern is scanned electronically.

1. Image formation in a structured illumination microscope (SIM)

The structured illumination technique relies on the fact that only the zero spatial frequency does not attenuate with defocus when imaging an object in a widefield microscope. This suggests that if the illumination system of the microscope is modified such that a grid pattern is projected onto the object, the microscope will only image the modulated part of the sample efficiently when it lies in the focal plane. As the object is moved out-of-focus, the grid pattern is strongly attenuated and eventually

disappears. An image is then obtained where the in-focus object is encoded by a sharp modulation pattern. To retrieve the modulated part of the object, a simple processing of at least three images, taken at three complementary grid positions, is required. This method has been demonstrated both in brightfield reflected light [14, 111] and fluorescence imaging [15, 16].

1.1. Structured illumination principles

1.1.1 *Microscope setup*

Fig. 4.1 shows the fluorescence microscope setup used for structured illumination where an optical grid mask has been positioned in critical illumination, so as to image it onto the specimen. The grid is uniformly illuminated by an incoherent light source placed in Kohler illumination. This light source can be either a conventional microscopy lamp or a broad area commercial LED. The image of the specimen is finally captured by a detector array, such as a CCD camera. When imaging a fluorescent sample, a dichroic mirror together with excitation and emission filters ensure that only fluorescence light is captured by the camera.

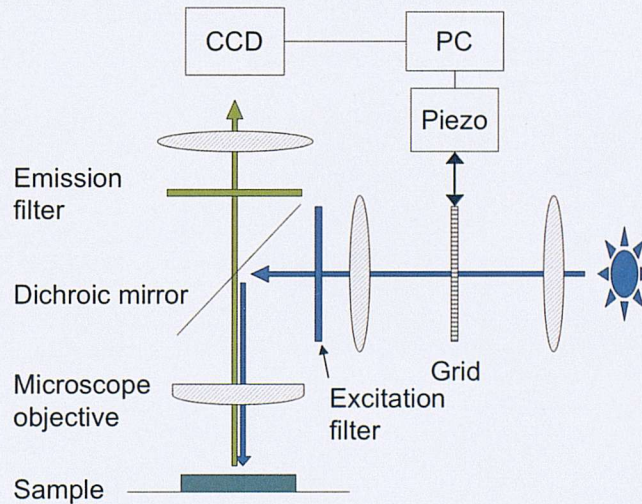


Fig. 4.1. Grid projection microscope setup.

Several image processing methods can be used to retrieve the modulated part of the object. For each grid position, an image is captured and the modulated raw images are then processed together. If N images are captured where the grid is moved by $1/N$ of a grid period, a sectioned image can be obtained by calculating

$$I_{\text{sec}} = \sqrt{\sum_{m \neq n} (I_m - I_n)^2} \quad (4.1)$$

If acquiring only three images I_1 , I_2 and I_3 in which the grid was shifted by a phase $\phi_1 = 0$, $\phi_2 = 2\pi/3$ and $\phi_3 = 4\pi/3$, respectively, this gives

$$\begin{aligned} I_{\text{sec}} &= \sqrt{(I_1 - I_2)^2 + (I_2 - I_3)^2 + (I_1 - I_3)^2} \\ &= \sqrt{2} \left| I_1 + I_2 \exp\left(-j\frac{2\pi}{3}\right) + I_3 \exp\left(-j\frac{4\pi}{3}\right) \right| \end{aligned} \quad (4.2)$$

Similarly, a conventional widefield image can be reconstructed by calculating

$$I_{\text{WF}} = \frac{I_1 + I_2 + I_3}{3} \quad (4.3)$$

1.1.2 Out-of-focus light rejection

The sectioning process is illustrated in Fig. 4.2 and Fig. 4.3. The first figure shows the three fluorescence raw images used to reconstruct a sectioned image of plant stem structure. Images were acquired using a home-built microscope with an Olympus UPlanFl 20x 0.5NA objective and an Orca ER CCD camera. An 80-micron pitch grating was Kolher illuminated using a Luxeon 1W blue LED and shifted using a piezo-actuator.

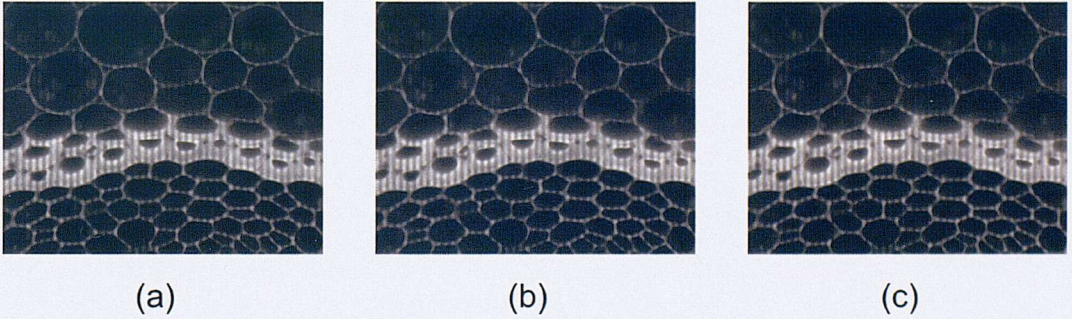


Fig. 4.2. Modulated raw images of a fluorescent plant stem structure.

Corresponding conventional and sectioned images are displayed in Fig. 4.3 both when the sample lies in and out-of-focus. It is clear that the structured illumination method allows for rejection of the out-of-focus background and provides sharp optical sectioning. This results in much sharper details when the object is in-focus and precise depth discrimination.

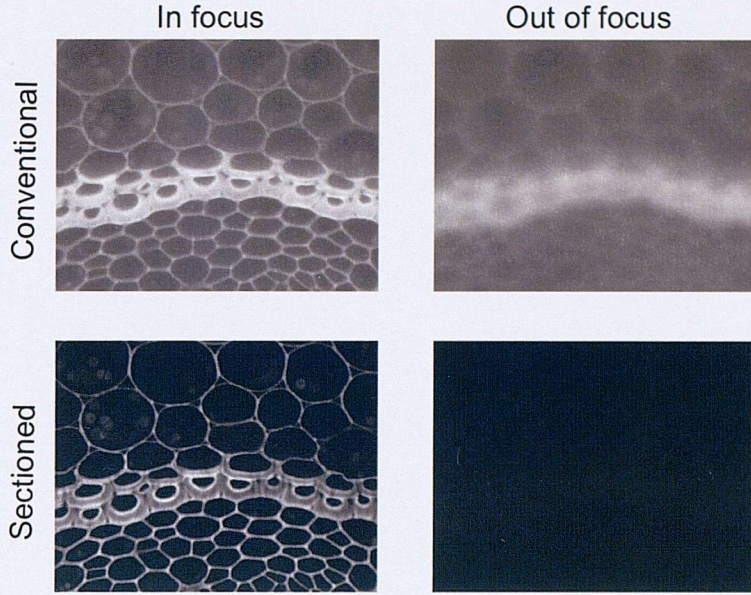


Fig. 4.3. Reconstructed conventional and sectioned images of a plant stem in and out-of-focus.

1.2. Fluorescence imaging with grid-projection structured illumination

1.2.1 *Image formation in a structured illumination microscope*

In a SIM, the object is illuminated by a modulation pattern obtained by imaging incoherently a one-dimensional grid. Assuming a perfect sinusoidal grid modulation orientated along w , the transmissivity of the grid can be written:

$$s_i(v, w, \tilde{\xi}) = 1 + \mu \cos(\tilde{\xi}v + \phi_i) \quad (4.4)$$

where μ denotes the modulation depth, ϕ_i represents an arbitrary grid phase, $\tilde{\xi}$ is the normalised spatial frequency of the grid expressed in optical units and related to the real spatial frequency ξ through

$$\tilde{\xi} = \xi \frac{M\lambda}{NA} \quad (4.5)$$

The variables λ and M denote the free-space wavelength of the excitation light and the microscope magnification, respectively. Conditions for which Equation (4.4) is valid when using a binary grid mask are discussed later. Normalized optical coordinates (v, w, u) were defined in chapter 2 and are related to the real coordinates (x, y, z) through

$$\begin{aligned}
 v &= \frac{2\pi n}{\lambda} \sin(\alpha) x \\
 w &= \frac{2\pi n}{\lambda} \sin(\alpha) y \\
 u &= \frac{8\pi n}{\lambda} \sin^2(\alpha/2) z
 \end{aligned} \tag{4.6}$$

When projecting the sinusoidal grid mask with an incoherent imaging system onto a specimen, the intensity of illumination, which illuminates the specimen, is given by:

$$\begin{aligned}
 I_{ill}(v, w, u; \tilde{\xi}) &= \iint s(v_1, w_1, \tilde{\xi}) |h(v_1 - v, w_1 - w, u)|^2 dv_1 dw_1 \\
 &= s(v, w, \tilde{\xi}) \otimes |h(v, w, u)|^2
 \end{aligned} \tag{4.7}$$

where $\otimes$ denotes the convolution operation and $|h(v, w, u)|^2$ is the intensity point spread function of an incoherent lens system. Introducing Equation (4.4) into Equation (4.7), the illumination pattern can be described as the inverse Fourier transform of the frequency response of the imaging system:

$$I_{ill}(v, w, u; \tilde{\xi}) = \iint S(s_x, s_y) T(s_x, s_y, u) \exp(2\pi j(s_x v + s_y w)) ds_x ds_y \tag{4.8}$$

where $T(s_x, s_y, u)$ represents the OTF of the incoherent imaging system and S is the Fourier transform of the grid pattern:

$$S(s_x, s_y) = \left[\delta(s_x) + \mu \frac{\delta\left(s_x - \frac{\tilde{\xi}}{2\pi}\right) + \delta\left(s_x + \frac{\tilde{\xi}}{2\pi}\right)}{2} \right] \delta(s_y) \tag{4.9}$$

Using the definition of the delta function, the illumination pattern can be expressed as [112]:

$$I_{ill}(v, w, u; \tilde{\xi}) = 1 + \mu T(|\tilde{\xi}|, 0, u) \cos(\tilde{\xi} v + \Phi_i) \tag{4.10}$$

The illumination pattern is still sinusoidal, but its modulation depth is attenuated with defocus according to the frequency of the grid used for projection. When projected onto a fluorescent object having a fluorescence generation function $f(v, w, u)$ and assuming the illumination and detection paths are the same, the modulated raw image $I_i(v, w, \tilde{\xi})$ captured by the camera can be described as

$$I_i(v, w, \tilde{\xi}) = \left[I_{ill}(v, w, u; \tilde{\xi}) \times f(v, w, u) \right] \otimes |h(v, w, u)|^2 \tag{4.11}$$

1.2.2 Optical sectioning

The microscope response to defocus is a measure of how fast the zero spatial frequency attenuates with defocus. It can be measured by scanning a fluorescent planar object, like a spin-coated fluorescent polymer sheet, through the focus of the objective. Considering a thin fluorescent object $f(t, w, u) = \delta(u)$ and using Equation (4.11), the modulated raw image captured by the camera can be written:

$$I_i(v, w, u; \tilde{\xi}) = 1 + \mu T^2(\tilde{\xi}, 0, u) \cos(\tilde{\xi}v + \Phi_i) \quad (4.12)$$

By acquiring three images shifted by $\Phi_i = 0, 2\pi/3, 4\pi/3$ and processing them using Equation (4.3), both the constant contribution and the remaining modulation are suppressed. An optically sectioned image is obtained, whose response to defocus varies as:

$$I_{\text{sec}}(u, \tilde{\xi}) = \mu T^2(\tilde{\xi}, 0, u) \quad (4.13)$$

Using Stockseth's approximation (see Appendix 1) for the incoherent OTF and normalising the function for $u=0$, the response to defocus can be written [14]

$$I_{\text{sec}}(u, \tilde{\xi}) = \left| 2g(\tilde{\xi}) \frac{J_1 \left[u\tilde{\xi} \left(1 - \tilde{\xi}/2 \right) \right]}{u\tilde{\xi} \left(1 - \tilde{\xi}/2 \right)} \right|^2 \quad (4.14)$$

This confirms that the grid projection technique exhibits optical sectioning on DC images, as does a confocal microscope. In the limit of $\tilde{\xi} = 0$, which is equivalent to removing the grid, $I_{\text{sec}}(u, 0)$ is a constant with defocus, as would be expected in a conventional widefield microscope. The maximum sectioning strength is obtained for a normalized grid frequency of $\tilde{\xi} = 1$. The grid visibility on a fluorescent sheet object is plotted in Fig. 4.4 for different defocus positions. Perhaps unexpectedly, it is the mid spatial frequencies that attenuate first when defocus increases. Fig. 4.5 shows the simulated axial response to a thin fluorescent sheet for a grid projection system. In the optimal case, the sectioning strength is about 6.5 optical units.

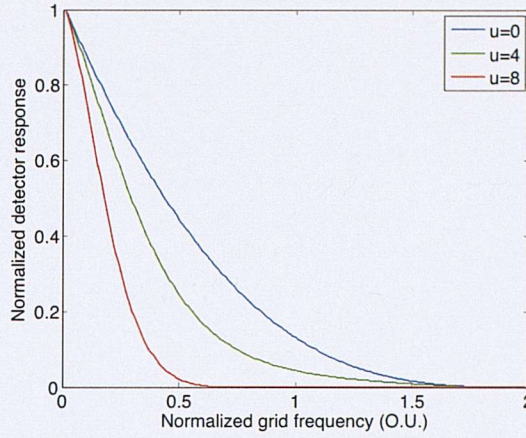


Fig. 4.4. Form of the function $T^2(|s|, u)$ as a function of $|s|$ for a variety of values of u .

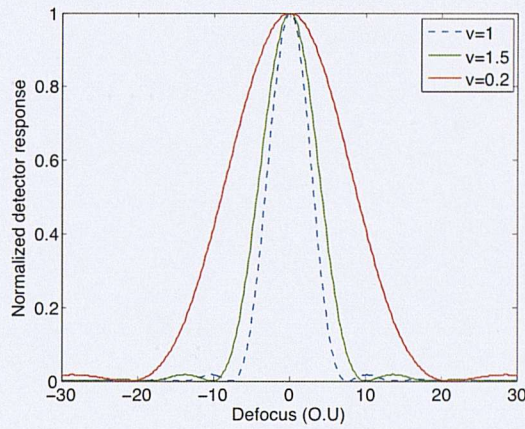


Fig. 4.5 Microscope response to defocus for several grid frequencies

Physical meaning of the sectioning strength variation

By measuring the FWHM of the normalized response with defocus curves for different grid frequencies, the axial response of the system is observed to degrade if the normalized frequency of the grid is either lower or higher than unity (Fig. 4.6). When the normalized grid frequency is smaller than unity, the grid becomes coarser and more defocus is needed to attenuate the image of the grid pattern, as illustrated in Fig. 4.4. The modulation penetrates further from the focal plane, which results in a lower sectioning strength.

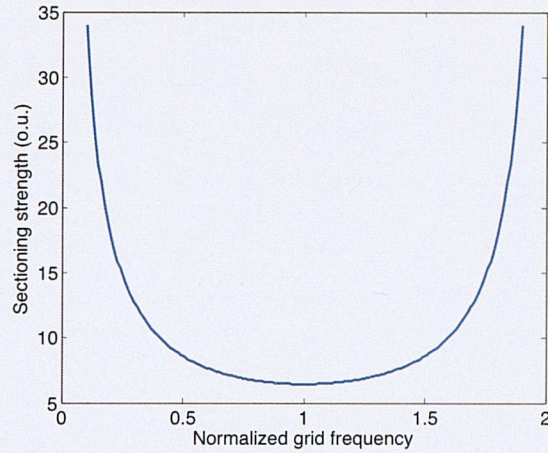


Fig. 4.6. Sectioning strength for different grid frequencies

When the normalized grid frequency is above unity, the grid becomes finer and finer and a degradation in sectioning strength can be seen again. To illustrate this latter case, let's consider the simple optical system shown schematically in Fig. 4.7.

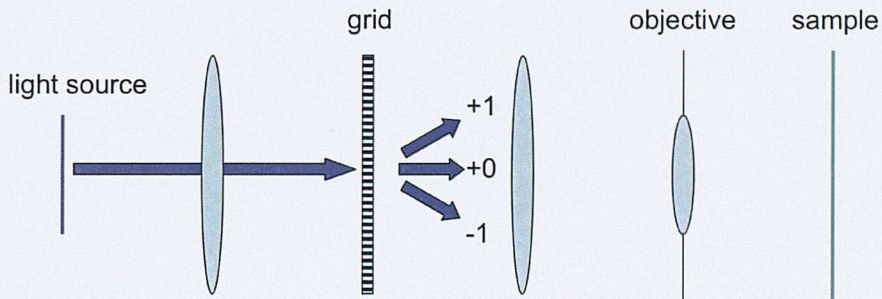


Fig. 4.7. Simple schematics of the grid projection system. The grid pattern is assumed to be sinusoidal such that only the -1 and +1 orders are diffracted.

Fig. 4.7 shows a schematic of the diffraction process when projecting a grid onto the sample. Supposing that the grid is purely sinusoidal and is illuminated by an incoherent extended light source positioned in Kohler illumination, three diffraction orders (-1, 0 and +1) will be generated on the pupil plane of the objective lens. The sinusoidal modulation in the image of the grid obtained in the sample plane eventually results from the interference between these three diffraction orders. Because the illumination is incoherent, only the portions of the light common to the +1, 0 and -1 orders that are captured by the pupil of the lens are mutually coherent and can interfere to reproduce the modulation pattern. The rest of the light cannot interfere and contributes to increasing the DC term in the image.

Fig. 4.8 tries to illustrate what happens for different grid spatial frequencies. The black dotted circle in Fig. 4.8 (a) represents the pupil of the objective lens. In Fig. 4.8 (b) and (c), the +1 and -1 diffraction orders are represented in blue. The 0th order is not represented but coincides with the objective pupil. From the diffraction theory, the +1 and -1 orders can be represented as a pupil shifted by a distance d from the centre of the objective pupil:

$$d = \pm \frac{\tilde{\xi} R}{2} \quad (4.15)$$

In the same figure, the red dotted circles represent the same pupils shifted by the distance $2d$. These red circles do not have a physical meaning, but help finding the mutually coherent regions of the +1, 0 and -1 orders. These regions are represented by the common areas between the pupil and the dotted red circles, whereas the mutually coherent part of the 0 order appears in dark blue. As the grid becomes finer and finer, the +1 and -1 orders are pulled apart, which results in both decreasing the surface of the mutually coherent areas and increasing the average distance of those areas to the centre of the objective pupil.

As a first result, the amount of light that can interfere decreases, which results in decreasing the modulation depth of the grid pattern. The modulation depth of the grid pattern for different grid frequencies is illustrated by the grid visibility for different spatial frequencies with no defocus, shown as the blue curve in Fig. 4.4. In the limit when $\tilde{\xi} = 2$, the mutually coherent areas become very small and practically no modulation can be observed.

Another consequence of using a very fine grid is that the coherence of the mutually coherent areas increases. The limit when $\tilde{\xi} = 2$ is equivalent to interfering three collimated laser beams at the sample, where the +1 and -1 beams are coming towards the sample plane at an angle equal to the full aperture angle of the lens. However, the resulting interference pattern, which is known as non-localised fringe pattern, has a very large depth of focus [29]. The modulation does not attenuate with defocus anymore, which results in a lower sectioning capability of the structured illumination microscope.

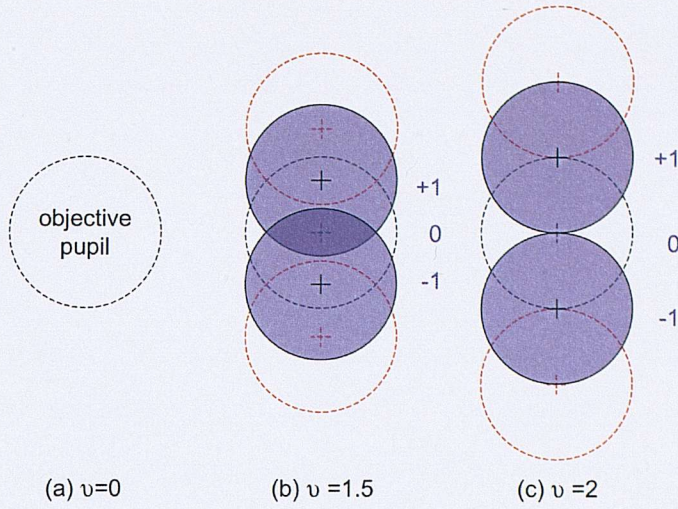


Fig. 4.8. Diffraction orders overlapping at the objective pupil for several normalized grid frequencies (v in the figure).

1.3. Structured illumination in practice

1.3.1 *Structured illumination using a square grating*

Because it is practically difficult to fabricate purely sinusoidal grids, structured illumination is usually implemented using a square grating to generate the single frequency modulation. As a square wave can be decomposed into a Fourier series having only odd terms, odd diffraction orders are present in the pupil plane of the objective lens. The raw image of the binary grid captured by the camera can be described as

$$I_i(v, u; \tilde{\xi}) = 1 + \mu \sum_{0 \leq k \leq \frac{1}{\tilde{\xi}} - \frac{1}{2}} \frac{1}{2k+1} T^2((2k+1)\tilde{\xi}, u) \cos((2k+1)\pi\tilde{\xi}v + \Phi_i) \quad (4.16)$$

The highest diffraction order $2N+1$ passing through the lens pupil is then given by

$$2N+1 = \text{abs}\left(\frac{2}{\tilde{\xi}}\right) \quad (4.17)$$

Then, as soon as $\tilde{\xi} \leq 2/3$, higher harmonics are projected onto the sample and can degrade the quality of the confocal image. Assuming a thin fluorescent object in focus ($u=0$), the table below summarizes the remaining modulation present in the sectioned image for several odd harmonics when processing the confocal image using Equation (4.2).

Harmonics	Sectioned image	Conventional image
3	I_{sec}	Third harmonics
5	I_{sec} + fifth harmonics	Third and fifth harmonics
7	I_{sec} + fifth + seventh harmonics	Third, fifth and seventh harmonics

Table 4-1: Residual modulations in the sectioned and conventional images using a three step structured illumination processing

With a three step processing, the third harmonics is eliminated in the sectioned image although it produces a faint residual modulation in the reconstructed widefield image. This modulation is barely noticeable in practice due to the third and higher order intensities being usually a tiny fraction of the DC component. In order to avoid any residual modulation in the sectioned image, a number of phase steps proportional to the numbers of the orders that gain admittance through the objective pupil should be used. For instance, if the fifth order is captured by the lens, a sectioned image with no artefacts would be produced using at least 15 steps, the third harmonics being also captured. This suggests that a grid having a normalized frequency greater than $\tilde{\xi} = 2/5 = 0.4$ is required when using the three step processing such that no residual modulation can be observed in the sectioned image. In practice, a normalized grid frequency between 0.5 and 1 is a good trade off between large modulation depth, thus high signal-to-noise ratio, and good sectioning capability. Below $\tilde{\xi} = 0.5$ the sectioning capability of the microscope degrades rapidly as seen previously.

For $\tilde{\xi} > 1$, besides degrading the sectioning capability of the microscope, Moiré aliasing effects appear as a result of the interference of the fine grid pattern with the high spatial frequencies present in the sample. The Moiré pattern takes the form of a lower spatial frequency pattern superimposed with the image of the object and can be used to double the resolution of the structured illumination system [67, 113].

1.3.2 *Artefacts resulting from an imperfect binary grid*

Due to defects in the binary grating, such as inaccurate mark-space ratios, second harmonics are often present in the grid spectrum. Using a three-step process, second

harmonics will result in a third harmonics residual modulation in the sectioned image. Important enough, because the illumination is incoherent the second harmonics cannot be optically filtered out and an image post-processing might be required. Indeed, in the limit when the second diffraction orders are pulled out of the pupil, mutually coherent area from the first orders disappear, as illustrated in Fig. 4.8 (c) where the red circles represent now the +2 and -2 orders. However, assuming the third harmonics does not enter the pupil ($\tilde{\xi} > 2/3$), the residual modulation can be avoided using four illumination steps according to the argument detailed earlier [114].

2. Performance of structured illumination in presence of photon noise

Because structured illumination is a technique that relies on differences between images as a modulation pattern is moved, it is susceptible to motion artefacts, grid position inaccuracy and non homogeneous photo-bleaching [115]. During the acquisition, a CCD camera captures raw images. This detector usually has a well defined and characterised noise performance that is often only limited by the photon shot noise of the captured signal. Because both the out-of-focus blur and in-focus image reach the CCD camera during acquisition, it is of interest to analyse the transmission of shot noise from the modulated raw images to the final confocal image.

2.1. Description of the photon noise transmission

2.1.1 *General equations*

In order to simplify the description of noise transmission, the linear form of Equation (4.2) will be used.

$$\begin{aligned} I_{\text{sec}} &= \sqrt{2} \left| I_1 + I_2 \exp\left(-j\frac{2\pi}{3}\right) + I_3 \exp\left(-j\frac{4\pi}{3}\right) \right| \\ &= \sqrt{2} |I_c| \end{aligned} \quad (4.18)$$

Where I_c is a complex sectioned image, which we use particularly in this analysis as it is a linear combination of the measured images and so noise is easier to calculate. Consider a general case where the object can have both in-focus and out of focus flat field components. In this most general case we can define the intensities detected in such an imaging system in the following terms:

$$I_n = I_b + I_f \cos(\tilde{\xi}t + \Phi_n) \quad (4.19)$$

In this modified version of Equation (4.4), Φ_n are the relative spatial phases of the three illumination patterns, the intensity I_b represents light both from out of focus regions as well as some contribution from the focal plane whereas I_f represents the modulated light that comes from only the focal plane. It should be noted that I_b must always be greater than I_f otherwise negative intensities will result. If I_n is measured in number of photons then each of the three I_n values is assumed to be independently normally distributed around its mean value $\langle I_n \rangle$ with a standard deviation of $\sqrt{\langle I_n \rangle}$ - a reasonable assumption for photon numbers greater than about 20 where shot noise is the limit.

We can calculate the probability of making a particular measurement of $I_c = x + iy$ from a series of measurements the I_n each with their own mean and standard deviation given by their Poisson statistics. The probability of making each measurement of the I_n as approximated by a Gaussian distribution can be defined as:

$$P[I_n = I_n] = \frac{1}{\sqrt{2\pi(I_b + I_f \cos(\tilde{\xi}v + \Phi_n))}} \exp \left[-\frac{(I_n - I_b - I_f \cos(\tilde{\xi}v + \Phi_n))^2}{2(I_b + I_f \cos(\tilde{\xi}v + \Phi_n))} \right] \quad (4.20)$$

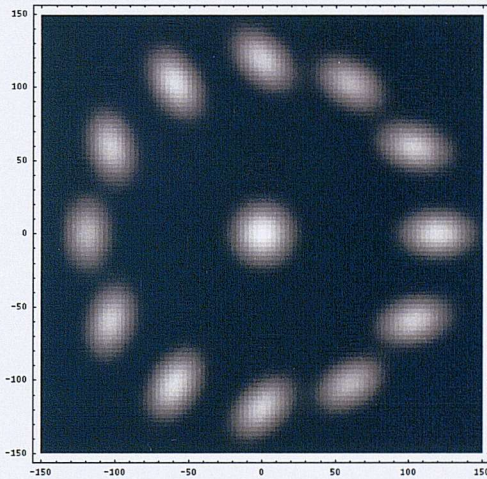
However there are several ways of choosing the I_n such that we achieve the same I_c so we must sum over all possible combinations. Once I_1 is chosen, then for a given $I_c = x + iy$, I_2 and I_3 are now fixed as it can be shown from Equation (4.18) above that $I_2 = I_1 - x + y/\sqrt{3}$ and $I_3 = I_1 - x - y/\sqrt{3}$. The probability of making these three measurements together is the product of making those measurements individually. To calculate the total probability of achieving our particular result I_c , that probability product simply needs to be integrated over all possible I_1 .

$$P[I_c = x + iy] = \frac{2}{\sqrt{3}} \int_{-\infty}^{\infty} P[I_1 = I_1] P[I_2 = I_1 - x + y/\sqrt{3}] P[I_3 = I_1 - x - y/\sqrt{3}] dI_1 \quad (4.21)$$

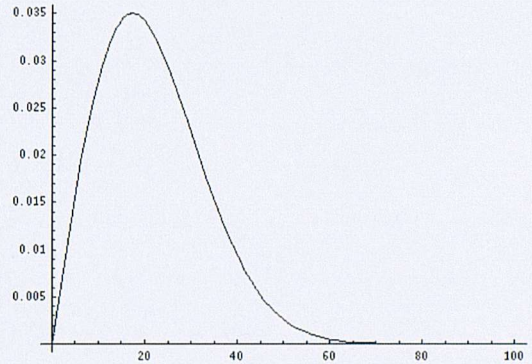
where the factor $2/\sqrt{3}$ maintains the correct normalisation. The resulting probability distribution function for I_c can thus be calculated (Equation (4.22)). As it is simply a

linear combination of the I_n , it is itself normally distributed in the complex plane as can be seen in Fig. 4.9 (a), although that distribution is anisotropic and depends on $\tilde{\xi}v$ explicitly.

$$P[I_C = x + iy] = \frac{2}{3\pi\sqrt{4I_b^2 - I_f^2}} \exp \left[-\frac{\left\{ \begin{aligned} &(4I_b - 2I_f) \left(\left(x - \frac{3}{2}I_f \cos(\tilde{\xi}v) \right) \cos\left(\frac{\tilde{\xi}v}{2}\right) - \left(y - \frac{3}{2}I_f \sin(\tilde{\xi}v) \right) \sin\left(\frac{\tilde{\xi}v}{2}\right) \right)^2 \\ &+ (4I_b + 2I_f) \left(\left(x - \frac{3}{2}I_f \cos(\tilde{\xi}v) \right) \sin\left(\frac{\tilde{\xi}v}{2}\right) + \left(y - \frac{3}{2}I_f \sin(\tilde{\xi}v) \right) \cos\left(\frac{\tilde{\xi}v}{2}\right) \right)^2 \end{aligned} \right\}}{3(4I_b^2 - I_f^2)} \right] \quad (4.22)$$



(a)



(b)

Fig. 4.9. Probability distribution functions of the sectioned measurements. (a) The probability distribution functions for I_C across the complex plane for a range of values of $\tilde{\xi}v$ (spaced by $\pi/6$ radians), for $I_b=100$ photons and $I_f=80$ photons (except in the centre where $I_f=0$). (b) The probability distribution function for I_S when $I_b=100$ and $I_f=0$. As can be seen the signal has a positive mean of about 21.7.

In order to evaluate the probability distribution function for I_{sec} two regions of interest can be considered. The first is when the mean value of I_C is significantly larger than the size of its distribution, i.e. when there is significant amount of in focus light. The probability distribution for I_{sec} can be approximated by the projection of that for I_C along the line joining the origin with its mean value. Under these

circumstances I_{sec} is also normally distributed but that the width of that distribution depends on $\tilde{\xi}v$ i.e. on the position in the image plane relative to the grid. This can be seen from the way the asymmetric distributions rotate in Fig. 4.9 (a) resulting in the noise being modulated at a spatial frequency 3x that of the grid illumination pattern. Performing the calculation one finds that I_{sec} is distributed with a probability given by

$$P[I_{\text{sec}}] = \frac{1}{\sqrt{3\pi(2I_b + I_f \cos(3\tilde{\xi}v))}} \exp \left[\frac{(I - 3I_f / \sqrt{2})^2}{3(2I_b + I_f \cos(3\tilde{\xi}v))} \right] \quad (4.23)$$

giving a mean $\langle I_{\text{sec}} \rangle = 3I_f / \sqrt{2}$, a standard deviation

$$\sigma[I_{\text{sec}}] = \sqrt{\frac{3}{2}(2I_b + I_f \cos(3\tilde{\xi}v))} \quad (4.24)$$

and therefore a signal to noise ratio of

$$\begin{aligned} \text{SNR}[I_{\text{sec}}] &= \frac{\langle I_{\text{sec}} \rangle}{\sigma[I_{\text{sec}}]} \\ &= \frac{\sqrt{3}I_f}{\sqrt{2I_b + I_f \cos(3\tilde{\xi}v)}} \end{aligned} \quad (4.25)$$

Most notable fact about this result is that the resulting noise is dependent on the position in the image plane and that therefore the signal to noise ratio can vary in the most extreme case when the sample is in-focus ($I_b = I_f$) from a value of $\sqrt{I_f}$ to $\sqrt{3I_f}$. This is also the case where the SNR is at its highest level. Any decrease in modulation I_f will result in a decreased SNR but lesser variation in that SNR across the image plane.

The second region of interest is where there is essentially no in-focus signal but significant out of focus signal (as in the centre of the complex plane in Fig. 4.9 (a) when $I_f = 0$). It can be shown that I_{sec} is now distributed with a probability given by

$$P[I_{\text{sec}}] = I \frac{\exp[-I^2/6I_b]}{3I_b} \quad (4.26)$$

as shown in for the case where $I_b = 100$. The most significant feature of this is that the mean value $\langle I_{\text{sec}} \rangle = \sqrt{(3\pi I_b)/2} = 21.7$ is greater than zero. Thus in the presence of significant out of focus light a DC offset is introduced into the sectioned image. There

is also noise in the signal, which is reduced because of the non-linear modulus function but has a value (standard deviation) of $\sqrt{(6 - 3\pi/2)I_b}$.

2.1.2 Simulations

A simple way of illustrating the noise distribution in the sectioned images is to perform a computer Monte-Carlo simulation of the structured illumination process. Starting with three sinusoidal modulation patterns with $I_f = 80$ and $I_b = 100$, Poisson noise is added to the raw images prior to sectioning. The process is repeated for 30 frames and the standard deviation is measured for each pixel across data set, as shown in Fig. 4.10. It can be checked that the standard deviation spans from $\sigma[I_{\text{sec}}] = \sqrt{3(100 - 80/2)} = 13.4$ to $\sigma[I_{\text{sec}}] = \sqrt{3(100 + 80/2)} = 20.5$ and is modulated at three times the grid frequency, as predicted by the theory.

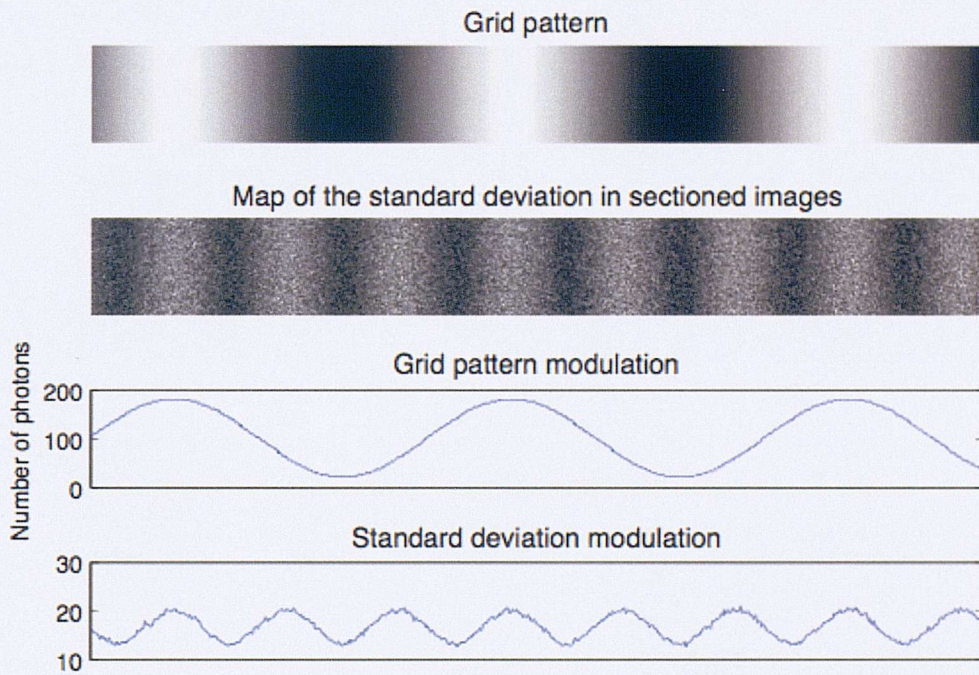


Fig. 4.10. Computer simulation of the structured illumination in presence of Poisson noise

2.2. Measurements

Fig. 4.11 shows experimental results for the measuring the noise distribution in the sectioned images. A thin fluorescent sheet (kindly provided by Paul Staviniou, Group of Solid State Physics, Imperial College London) was placed in focus and illuminated with a grid. A set of 90 images was acquired to obtain 30 sectioned images using the

three-step image processing. The integration time of the camera was set such that most of the dynamic range was used to ensure the measurements were photon-noise limited.

Fig. 4.11 (a) shows a modulated raw image that was used to reconstruct the average conventional (Fig. 4.11 (b)) and average structured illumination (Fig. 4.11 (c)). The noise distribution was obtained by calculating the standard deviation across the data set for each pixel. A third harmonic structure can be observed in the noise distribution, although some of the deviation might be due to a second harmonics present in the grid pattern and generating a residual third harmonics in the sectioned images.

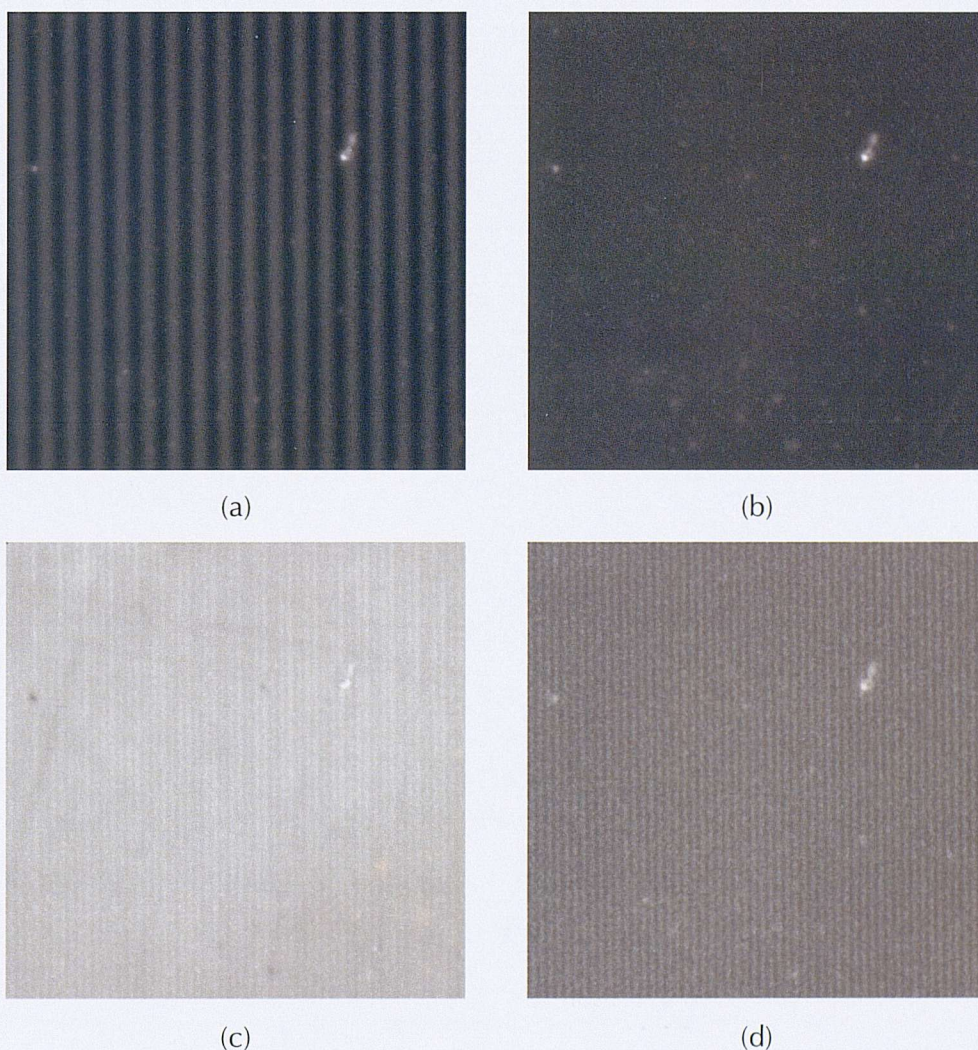


Fig. 4.11. Experimental images for 100nm fluorescent polymer film: a) Raw modulated image, b) Conventional image, c) Sectioned image and c) Distribution of noise from a set of 30 sectioned images

The performance of a structured illumination microscope can be evaluated when the measured signals are limited by photon shot noise. The analysis shows that artefacts can be introduced as a result of this. Firstly the noise in the sectioned image can itself be modulated at three times the grid frequency when the in focus signal is strong. Secondly, when the in-focus signal is weak an offset can be introduced in the sectioned image due to the noise in the out of focus signal. These results may have implications for using such techniques in quantitative or ratiometric fluorescence techniques such as fluorescence lifetime, Ca²⁺ or FRET imaging.

3. Structured illumination using a stripe array micro-LED

Structured illumination with no moving parts was implemented by replacing the grid and the external light source by a blue micro-stripe array. The performance of the new microscope is measured and device improvements required to obtain a reasonable image quality are discussed.

3.1. Microscope setup

Fig. 4.12 shows the experimental microscope configuration used to produce optically sectioned images. The micro-stripe array is located in the illumination path of an upright Olympus BX41 microscope in a critical illumination configuration so that the illumination pattern of the LED array was imaged directly onto the sample.

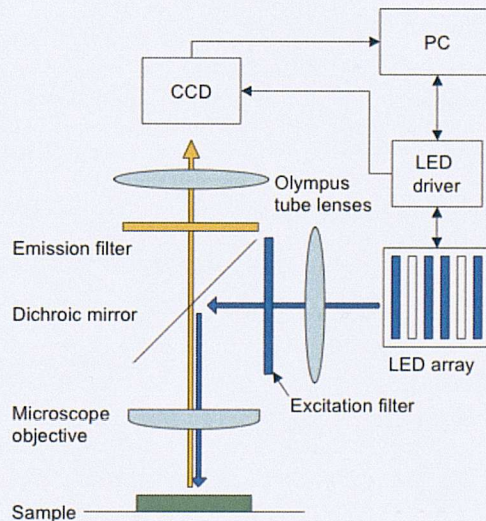


Fig. 4.12. Microscope setup

The optics in the illumination path were removed and replaced by a standard 180mm Olympus tube lens in order to avoid aberrations when projecting the LED pattern onto the object. The LED device would thus illuminate a field of $3.6/M \times 4.08/M$ mm on the sample, where M is the magnification of the microscope objective used. Fluorescence gathered by the objective forms an image on an Orca ER CCD camera. Because the objective is used both to illuminate and to image the sample, the magnification of the pattern image onto the camera does not vary when microscope objective is changed but only depends on the ratio of the focal lengths of the illumination and imaging tube lenses. In our actual setup both tube lenses are conventional Olympus 180 mm tube lenses so this ratio is 1.

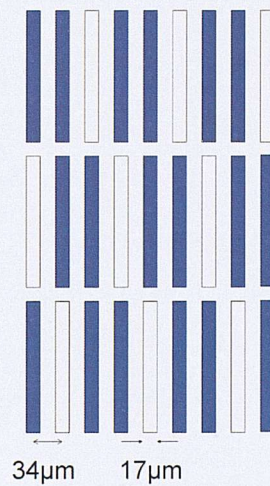


Fig. 4.13. Scanning scheme used for grid-projection

Fig. 4.13 shows how the three different grating positions were achieved by successively shifting a three-stripe repeat grid pattern by one stripe at a time. The grid used is the smallest possible with this device at 102 μm , although more complex scanning schemes can also be achieved using more than three LED stripes and hence longer periods. This solid-state scanning approach has the advantage of removing the possible inaccurate grating shifts that give rise to artefacts in the sectioned image [115], but does suffer currently from introducing higher grid harmonics into the illumination pattern that can also result in artefacts in the sectioned image.

3.2. Performance

3.2.1 *Images*

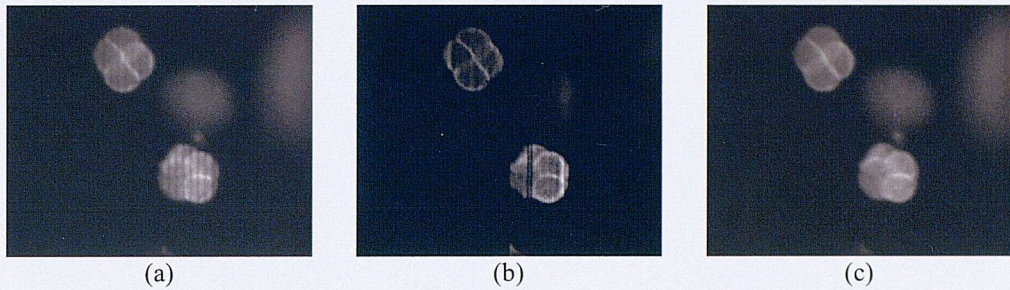


Fig. 4.14. 20x images of stained pollen grains acquired with grid-projection structured illumination. (a) Modulated raw image, (b) Sectioned image, (c) Conventional image

Fig. 4.14 illustrates the structured illumination technique applied to optically section stained pollen grain. The pollen grain images were acquired using a 20x 0.5NA Olympus objective, with 500 ms exposure per image and 3 mA current per LED stripe. In the image sequences shown in Fig. 4.14 (a) we can see that light from pollen grains lying out-of-focus is not modulated whereas that from the in-focus grains is strongly modulated. In the sectioned image, Fig. 4.14 (b), the out-of-focus objects are clearly suppressed compared to the conventional image, Fig. 4.14 (c).

Residual artefacts are presenting the form of a high frequency modulation across the image. These are similar to those predicted above and are due to a mix of both odd and even higher harmonics present in the illumination pattern. This can potentially be reduced by using a finer LED line array structure with more LED elements per grid period. Other artefacts are clearly visible due to imperfections in the array such as missing and shorted elements, which can obviously be removed by improved device fabrication.

3.2.2 *Sectioning strength*

In order to demonstrate and quantify the sectioning ability of our microscope, we simulated and then measured the detected signal in the confocal images as a thin fluorescent sheet is scanned through the focus of the microscope objective. Such an experiment provides a good indication of the ability of the system to reject out-of-focus light as it is only this zero spatial frequency that is not sectioned in even conventional microscopes, and which contributes most to out-of-focus blur, as described previously. For these simulations and measurements we used a 40X

Olympus UPlanFI 0.75 NA air microscope objective together with the corresponding Olympus tube lens and an appropriate Olympus blue filter cube. The images were captured with a Hamamatsu firewire ORCA ER camera triggered by the LED driver board, and the fluorescent sheet was moved from $-10\mu\text{m}$ to $+10\mu\text{m}$ in $0.2\mu\text{m}$ steps. Experimental axial response curves were calculated by integrating the total signal across the sectioned image as a function of defocus. The simulations are based on paraxial imaging theory and are only an approximation for such a high NA, but still show good agreement with experimental measurement.

The results of this calculation are plotted in Fig. 4.15 along with measured values from the microscope as described above. Good agreement between the experimental and the theoretical defocus response can be seen clearly. There is an offset in the measured signal, which can be attributed to device imperfections such as missing and shorted lines and resulted in isolated image artefacts that are not sectioned. The defocus response half width half maximum was measured to be $0.94\mu\text{m}$ while the theory predicts a HWHM width of $0.83\mu\text{m}$ for this $102\mu\text{m}$ grid and blue illumination, $\lambda=470\text{nm}$.

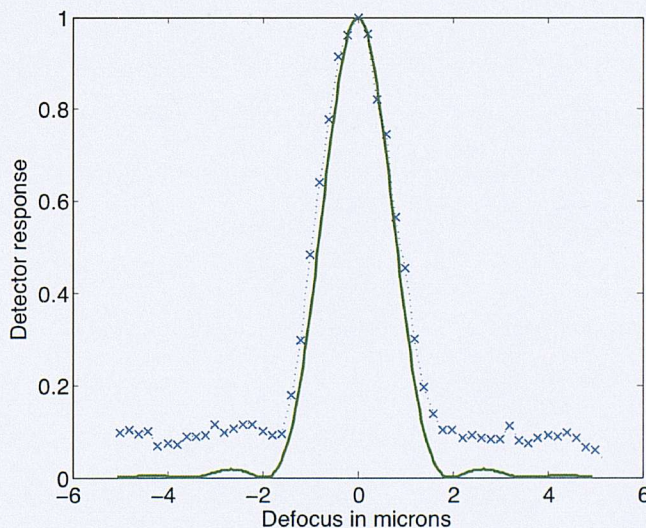


Fig. 4.15. Theoretical and experimental axial responses of the structured illumination system

3.3. Artefacts and future improvements

It is clear from Fig. 4.14 (b) that strong artefacts are present in the sectioned image. These artefacts are due to non-uniformities in the micro-stripe light source, such as broken LED stripes and even spatial frequencies present in the grid pattern. Higher

harmonics can be removed by Fourier-filtering the sectioned image. Missing stripes result in a brighter area in the sectioned image as the sample was not lit up at this location. This artefact can also be explained as a dramatic degradation of the sectioning strength at the missing stripe positions due to a much lower spatial frequency illumination.

In order to suppress those artefacts when using a micro-stripe LED, dramatic improvements in the emission uniformity will have to be made. First, a uniform emission along each individual emitter will have to be engineered. Flip-chip technology might be help for this purpose. Second, missing stripes will have to be avoided as far as possible. Manufacturing the LED device on a large scale using an automated industrial process should prevent such defects. Finally, diffractive micro-optics could be integrated on top of the emitters in order to filter out higher spatial frequencies.

4. Summary

The structured illumination method was presented that renders possible to achieve optical sectioning with minimal modifications of a conventional fluorescence microscope. The performance of the system was described in presence of normally distributed photon noise. The error in the optically sectioned images was found to be modulated at three times the frequency of the illumination pattern, which should be taken into account when performing quantitative confocal measurements. This error was also shown to be highly dependent on the intensity of the out-of-focus background, as the detector captures the out-of-focus light before rejecting it. A new way of implementing grid projection structured illumination was introduced that uses the micro-stripe LED technology described in a previous chapter. This system has numerous advantages in terms of simplicity, cost, and potentially speed, although problems in the uniformity of the device emission have a dramatic impact on the sectioned image quality.

Chapter 5 Line scanning confocal microscopy

The previous chapter showed how the stripe geometry LED array device could be used to produce optically sectioned images in a grid projection structured illumination geometry. That technique has obvious advantages in terms of speed and ease of processing. However, it relies on measuring intensity differences between images, and so it has clear drawbacks in terms of noise and imaging artefacts. Conversely, in a confocal microscope the detection slit blocks the out-of-focus light, which does not contaminate the confocal measurement. This physical rejection of the blur gives better results than the structured illumination technique when imaging deeper into scattering samples like organ tissues for example. This chapter explores the use of the same micro-stripe LED device in a line scanning confocal geometry.

First the line array is used with a CCD camera detector and various post-processing algorithms to implement a simple solid-state line scanning confocal microscope with no moving parts. This approach is shown to produce better images in terms of artefacts than the grid projection structured illumination system, but at a cost of slower acquisition times and poorer sectioning strength.

The first of these can be greatly improved by increasing the number of lines that are scanned in a parallel line confocal geometry. The acquisition rate is increased by a factor equal to the number of parallel lines scanned, but at the expense of an increasing non-confocal background signal in the image and additional line artefacts due to non-homogeneous illumination.

Finally a modified detection scheme is proposed that increases the suppression of out-of-focus signals in these line-scanning geometries using a subtractive post-processing step. This approach is demonstrated in both single and multi-line geometries, showing significantly improved optical sectioning and allowing superior correction of the line artefacts present in the slit confocal image.

1. Line scanning microscopy using a micro-stripe array

In the Minsky description of the confocal principle [1], the specimen is illuminated with a point source and a pinhole is used for detection. Although this arrangement provides superior out-of-focus light rejection, it was limited to imaging fixed specimens and could not achieve optically sectioned imaging in real-time. A decade after Minsky's invention several developments started to replace the point illumination by a line illumination and confocality was achieved by using a detection slit, in an attempt to image the human cornea in real-time [4, 6, 116]. In this manner, the original point-by-point image acquisition is replaced by a much faster line-by-line acquisition. With a single sweep of the scanning mirror, a whole optical section is imaged. Hence, the scanning speed increases by approximately the number of points that can be excited in parallel, which can be easily as high as 500. As described in Chapter 2 there is a price to be paid for this increase in speed: a decrease in optical sectioning power. In fact in the dimension parallel to the axis of the detection slit, the confocal principle is sacrificed, which results in a decrease by a factor of approximately 1.6 in axial resolution.

So far, slit scanning confocal systems relied on a fixed illumination and detection slits and the excitation beam is scanned across the sample by using a mirror arrangement. Several ways of descanning the fluorescence emission onto the fixed detection slit were invented, the easiest of which being for the fluorescence light to reflect back on the same scanning mirror towards the confocal detection. By using the stripe array LED as the fluorescence excitation source and a CCD camera to record the fluorescence signal, a new approach is investigated where the excitation beam is scanned electronically and the confocal image is reconstructed in software.

1.1. Microscope setup and image acquisition

The optical setup is the same as that used to perform structured illumination, as described in the previous chapter. The array is placed in critical illumination configuration so that the line pattern is imaged onto the object, as shown in Fig. 5.1. An image is captured on a 2-dimensional array detector for each line position, with no need to descanner the fluorescence light returning from the object. The light originating from the out-of-focus region of the object is rejected by post-processing the stack of

$N=120$ images corresponding to the N stripe positions and a confocal image can be reconstructed numerically.

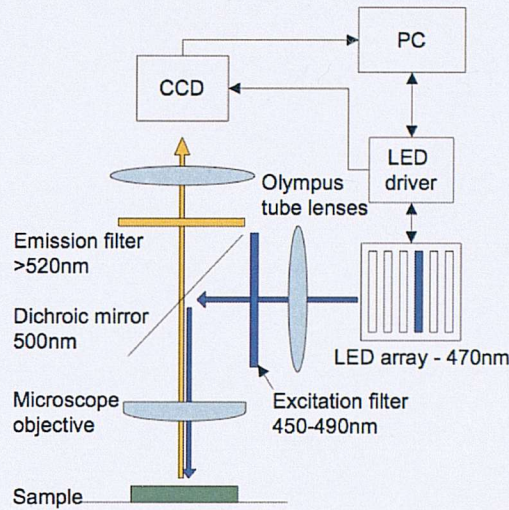


Fig. 5.1. Single line scanning microscope setup

During the acquisition, the computer triggers the LED board for each line position by sending an 8-bit command through the USB port. A stripe LED is turned on and the driver board synchronizes the image capture with the LED exposure time, which is previously saved into the board RAM memory. As the diode is turned off the image is read-out from the camera frame-grabber to the computer. This process is repeated for the N line positions to build up a full frame sectioned image. Triggering the image capture from the driver board allows for a precise control of the synchronisation between diode exposure and camera integration time in order to limit photo-bleaching of the sample. Triggering the driver board from the computer makes sure a raw image has been fully read-out before the next exposure and acquisition is triggered. Processing of the confocal image can either take place in real-time on the host PC, or after all N lines have been projected onto the object. The latter alternative avoids introducing an additional computational overhead during image acquisition.

1.2. Confocal detection using image post processing techniques

Two alternative post-processing techniques can be used to process the stack of raw images into a sectioned image and reject the out-of-focus light. The first approach is to mimic the confocal detection slit by implementing a set of virtual slits in software for each line position. For each illumination position, the fluorescence signal is masked

using the corresponding detection slit. The other approach consists of excluding the out-of-focus light at a pixel level by projecting for each pixel the maximum intensity of the stack of raw images [117]. This processing results in a confocal-like image because the in-focus object is likely to be brighter than the out-of-focus blur under patterned illumination.

1.2.1 Confocal detection using a virtual slit

Because a CCD camera is an array of point detectors, a simple way of implementing the slit confocal detection is to create a binary mask in software for each stripe position. By multiplying the captured image by the corresponding mask image, the out-of-focus light is physically rejected using this virtual confocal slit. The confocal image is finally calculated by summing the $N=120$ masked images. This reconstruction can be described by the equation:

$$I_{\text{sec}} = \sum_{i=1}^N \text{Slit}_i \cdot I_i, \quad (5.1)$$

where I_i is the image captured when illuminating the sample with the i^{th} LED stripe and Slit_i is the corresponding binary mask.

In order to create the set of binary masks, a set of N reference images is obtained by imaging a thin and homogenous polymer film or an in-focus mirror under single stripe illumination. From this set of reference images, several methods can be used to determine the corresponding set of binary masks, including thresholding and edge detection techniques. The simplest of these is just to detect for each pixel the position of the maximum intensity through the stack of N reference image. When a camera pixel belongs to the area illuminated by a stripe emitter, the intensity will be maximum, whereas it will be lower when illuminating the sample with the adjacent LEDs. The i^{th} mask can then be described as the group of pixels having the same maximum position through the stack of reference images, that is to say

$$\text{Slit}_i(x) = \begin{cases} 1 & \text{if } \text{MAXPOS}_z(x) = i \\ 0 & \text{else} \end{cases}, \quad (5.2)$$

where $\text{MAXPOS}_z(x)$ denotes the index of the maximum along the stack z -direction for the pixel x . This method is insensitive to sharp brightness variations along a single stripe, is computationally fast and attributes each pixel in the image to a slit mask. As

shown in Fig. 5.2, the detection of the maximum positions results in slits whose width might no be constant along their length.

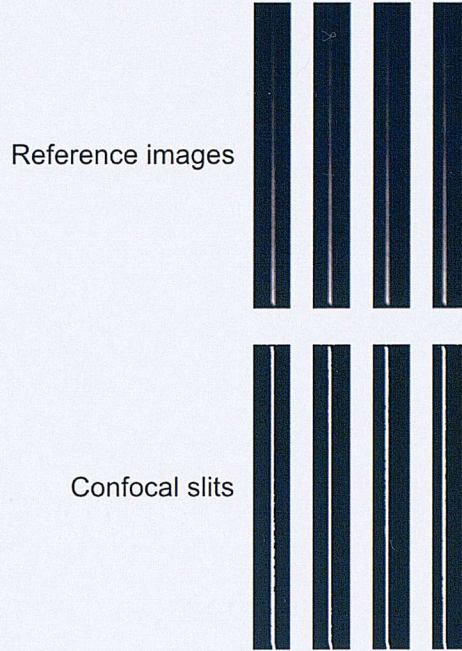


Fig. 5.2. Set of confocal slits by detection of the maximum positions

For the stripe devices described in chapter 2 with a 17 μm LED width on a 34 μm centre to centre spacing this will result in an effective 17 μm illumination slit and, on average, a 34 μm detector slit width. This method of generating virtual detection slits is independent of the precise array detector geometry, providing the detector pixel size is fine enough, and can be applied whatever the orientation of the illumination stripe array with respect to the array detector.

1.2.2 Confocal detection by maximum projection

A second post-processing confocal reconstruction method [117] was also implemented in which a confocal image is calculated from a stack of N object images without passing the detected fluorescence light through a detection slit, virtual or otherwise. In this method N wide field images of the object are acquired, one for each stripe position as before, and the maximum intensity from those N images is chosen for each image pixel. The sectioned image can thus be described by the equation:

$$I_{\text{sec}}(x) = \underset{x \in [Nx, Ny]}{\text{MAX}}_{i=1}^N [I_i(x)] \quad (5.3)$$

where $MAX[]$ denotes the maximum value from a set of pixels. Similarly, a wide field image I_{WF} can be calculated by averaging the N images.

$$I_{WF}(x) = \frac{\sum_{i=1}^N I_i(x)}{N} \quad (5.4)$$

In the maximum projection technique no detection slit is required making the implementation very straightforward. The sectioning capability comes here from the fact that in-focus parts of the object are usually brighter than those out-of-focus when illuminated with patterned light. However, because the light originating from an out-of-focus object is not physically rejected, it can still contribute to the maximum projection image when there is no in-focus signal. Also, this technique tends to have a larger background than that of the auto-slit confocal as it will always choose the brightest value from a set of noisy pixel samples.

Although the maximum intensity projection technique is rigorously equivalent to the confocal detection technique when imaging an in-focus object, the contribution of out of focus light and out-of-focus light from 'neighbouring stripes' can also lead to distortion when the amount of out-of-focus light is significant. However, the intensity of the distorted part of the image is usually very weak compared to the in-focus contribution and is barely noticeable in practice.

1.2.3 System performance

Images

The same cluster of stained pollen grains as used to test the structured illumination system is imaged with both these line-scanning techniques (Fig. 5.3). Experimental conditions are the same as previously. In both cases the same set of 120 images was used, each of which was acquired for approximately 10ms at an LED stripe current of 30 mA. Fig. 5.3(a) is a reconstructed conventional image (calculated by summing all 120 images) clearly showing large out-of-focus pollen grains. Fig. 5.3(b) and Fig. 5.3(c) show two optically sectioned confocal images processed using the maximum projection and the automatic slit detection methods respectively.

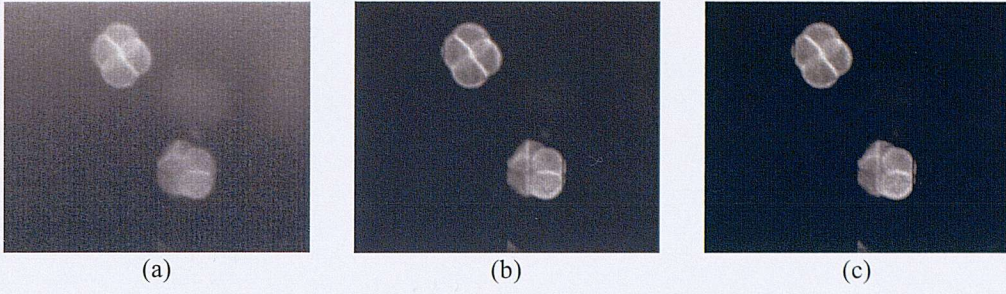


Fig. 5.3. 20x magnification images of stained pollen grains (a) conventional image, (b) maximum projection confocal image and (c) automatic slit confocal image.

Sectioning strength

For both line-scanning schemes described above, the sectioning strength for a fluorescent sheet should be identical and can be modelled by considering the incoherent imaging of the illumination slit onto the sheet object followed by the incoherent imaging of the resulting fluorescent image onto the detector slit. The sectioning strength can then be calculated by summing all the light passing through the detector slit. This can be calculated by an integral in Fourier space across the product of the illumination and detection lens 2-D incoherent optical transfer functions multiplied by the Fourier transform of the illumination and detection slits. Assuming an aberration-free system where both illumination and detection lenses are identical and excitation and emission wavelengths are the same, the defocus response of a fluorescence slit microscope under incoherent illumination can thus be written as:

$$I_{slit}(u) \propto \int_{-\infty}^{+\infty} T(s_x, u)^2 \operatorname{sinc}\left(\frac{ps_x}{2\pi}\right) \operatorname{sinc}\left(\frac{qs_x}{2\pi}\right) ds, \quad (5.5)$$

where p and q are the normalized width of the illumination and detection slits, respectively:

$$(p, q) = \frac{2\pi}{\lambda} (D_i, D_d) \sin \alpha \quad \operatorname{sinc}(x) = \frac{\sin(x)}{x} \quad (5.6)$$

The incoherent OTF $T(s, u)$ can be approximated again from Stokseth, as described in chapter 2. The results of this calculation are plotted along with experimental data recorded using automatic virtual slit detection. Again good agreement between theory and experiment are seen apart from a small offset ($\sim 5\%$) in the experiment, which in this case can be attributed to the light scattered from the back of the LED device, that has no structure in the image plane.

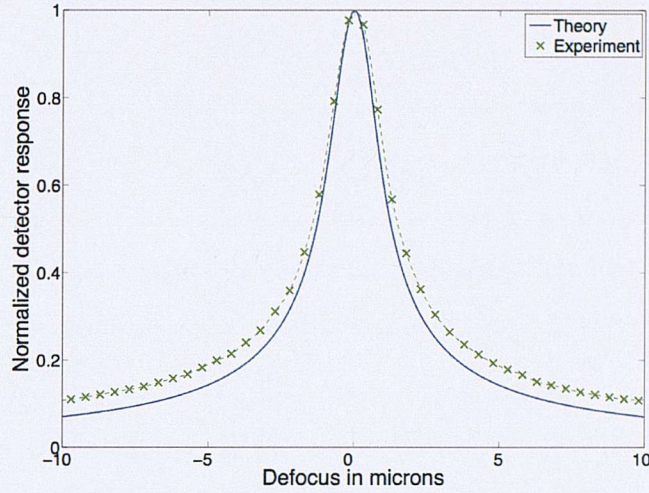


Fig. 5.4. Theoretical and experimental sectioning strengths of a slit scanning confocal microscope.

Using a 40X Olympus UPlanFI 0.75 NA air microscope objective together with the corresponding Olympus tube lens, an appropriate Olympus blue filter cube and an ORCA ER CCD camera, the experimental measurements indicate a FWHM response of $3.0\mu\text{m}$, while the theory suggests a FWHM of $2.6\mu\text{m}$. Both graphs also show significant out-of-focus tails, which, along with the large FWHM, indicate the poorer sectioning strength of line scanning techniques compared to grid projection. Processing the z stack of raw data both using the maximum intensity method and the virtual slit method gives, as expected, very similar answer as shown in Fig. 5.5.

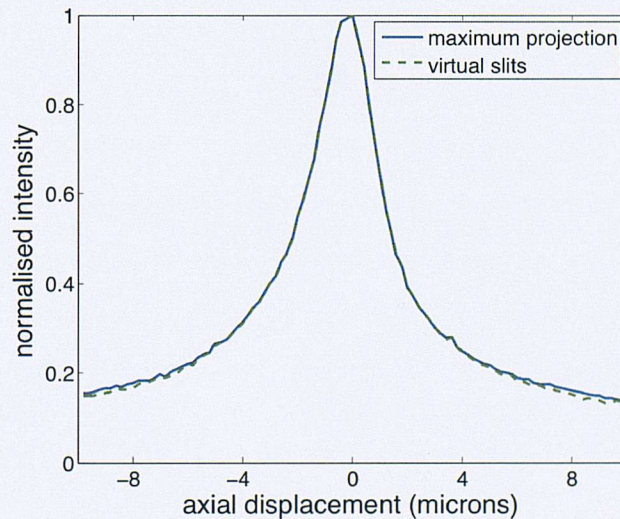


Fig. 5.5. Slit microscope responses to defocus using the maximum projection and virtual slits processing methods

1.2.4 Advantages and drawbacks of confocal post-processing methods

A clear advantage of these two confocal processing methods is the extreme simplicity of the detection arrangement. No physical detection slit is required to reject the out-of-focus light and the confocal image can be processed in real time, as these methods are not computationally intensive. Both detection schemes are also independent of the orientation of the LED array with respect to the camera. In addition photo-bleaching is reduced to its bare minimum as the sample exposure time is synchronized with the image capture.

However, because 120 full frames need to be read-out in order to reconstruct the confocal image, the overhead associated with reading out each image into the host computer meant that it took approximately 10 seconds to acquire the full set of images, for a total exposure of approximately 1.2 seconds. This suggested in particular that selective region-of-interest read-out such as is currently possible with CMOS cameras, would significantly increase the speed of the line scanning technique. Selective ROI readout is also possible with CCD cameras but it was too slow to implement. The following section describes how this approach might be implemented with an appropriate CCD detector system.

1.2.5 Confocal detection by fast CCD sensor windowing

Instead of relying on post-processing using virtual slits, haze rejection could be carried out directly at the camera sensor level by reading out regions of interest (ROI). Windowing the sensor allows for disregarding the out-of-focus information and transferring to the computer a limited number of pixels corresponding to the in-focus object only. As the illumination is scanned through the object, the position of the acquisition window changes to match the position of the illumination beam. However, because the acquisition window needs to be modified between two consecutive frames, one should only use cameras with fast windowing options, where the window parameters can be loaded quickly into the camera frame-grabber, in order to keep the delay introduced by changing the window parameters as small as possible.

The previous ORCA ER camera takes about 500ms to load the new window position in the camera frame-grabber. An additional overhead of 60 seconds would then be introduced only due to the camera reprogramming, which is still bigger than

the time reduced by reading out only a small portion of the sensor. To date no commercially available CCD camera has been developed with fast windowing capabilities, although no fundamental limitations arise from the sensor architecture. A dedicated driver could easily be engineered that allows for fast window reprogramming between two consecutive frames.

A readout scheme is proposed in Fig. 5.6 to take advantage of the CCD camera architecture and should allow for tens of confocal images per second. Only the pixels corresponding to the detection slit are vertically binned when shifted into the sensor buffer and then read-out. Other pixels containing the out-of-focus information are disregarded and only the right pixels are read-out. Assuming a slow 10MHz readout rate and a 1000 x1000 sensor, 120 x1000 pixels only will have to be read-out from the camera and transferred from the frame-grabber to the computer in order to reconstruct a single confocal image, which could be achieved as fast as 12ms per frame. After each frame is transferred, a new window position is loaded into the camera while acquiring the next frame. This new windowing feature could be easily implemented when changing the camera driver.



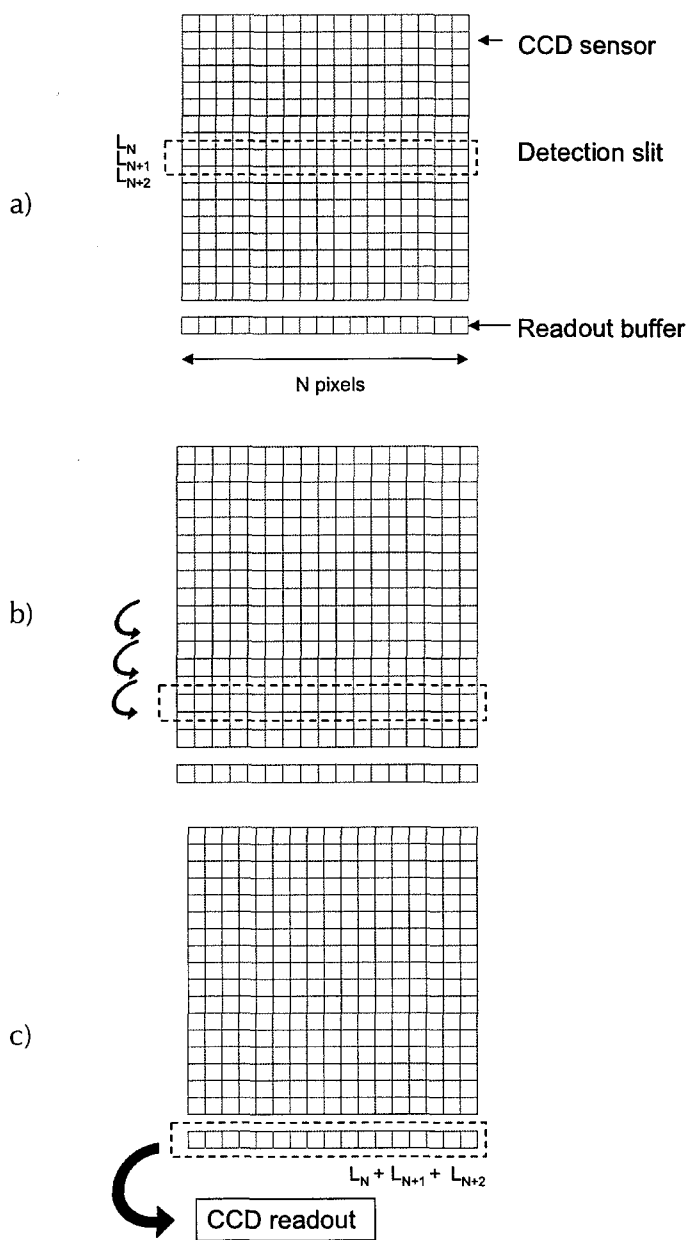


Fig. 5.6. CCD readout scheme for fast line scanning microscopy, including the steps of (a) calibration of the illumination position, (b) elimination of the disregarded pixels and (c) binning of the rows of interest before readout.

2. Multiple line scanning microscopy

In order to further increase the acquisition speed of the slit microscope, several schemes were proposed that scanned multiple lines in parallel on the sample. The microscope setup remains the same as described earlier (Fig. 5.1) but images are processed differently. Processing for multi-stripe detection was incorporated in the same graphical interface as for the structured illumination and single line scanning techniques, which allowed for a versatile microscope environment, and was fully automated to produce confocal images in real time. Assuming a separation of N lines between two consecutive beams in the illumination pattern, only N images need to be acquired to reconstruct a confocal image. As the number of lines in the illumination pattern increases, cross talk becomes more and more prominent, which results in a lower haze rejection and a degraded sectioning strength. Again several detection schemes were implemented and the sectioning capability was measured for different microscope setups.

2.1.1 Multiple line detection schemes

Confocal detection using the maximum projection method for all pixels in the N raw images can be readily used as described previously. Because the out-of-focus rejection takes place at a pixel level, the result is a confocal image with higher background due to cross talk.

Using a similar principle as before, confocal images can also be obtained by masking the raw images using a multi-slit binary mask. From the same set of binary slits obtained by imaging a homogenous fluorescent sheet or a mirror under single stripe illumination, a multi-slit detection mask $Mask_i$ can be created for the i^{th} illumination position using the formula:

$$Mask_i(x) = \sum_{0 \leq j \leq \frac{120}{N}} Slit_{(i+N \times j)}(x), \quad (5.7)$$

where $Slit_i$ denotes the binary mask obtained by imaging a uniform reference object under the single i^{th} stripe illumination. The confocal image can then be calculated using the equation:

$$I_{conf}(x) = \sum_{i=1}^N Mask_i(x) \cdot I_i(x). \quad (5.8)$$

Similarly, a widefield image can be reconstructed by summing the raw images:

$$I_{WF}(x) = \frac{\sum_{i=1}^N I_i(x)}{N} \quad (5.9)$$

Fig. 5.7 illustrates how confocality is sacrificed as more and more beams are scanned simultaneously across a sample of *Convalaria* plant stem. These images were acquired at 30mA per stripe using a 40x 0.75NA dry objective with an Olympus blue cube. The sample was exposed during 100ms per illumination position, resulting in a total exposure time of 1s when scanning 12 beams simultaneously (Fig. 5.7.d).

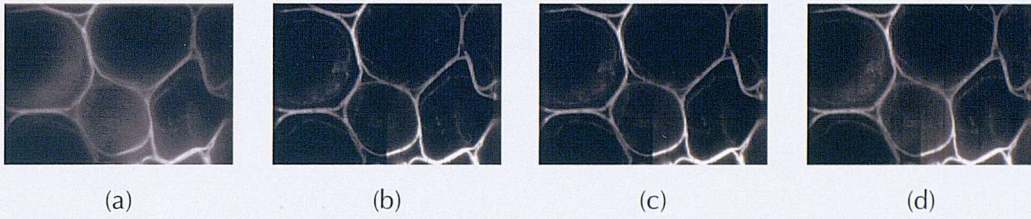


Fig. 5.7. Multi-beam confocal fluorescence images of a plant stem using a micro-stripe LED. (a) Reconstructed widefield image, Confocal images obtained by scanning (b) a single beam, (c) 4 beams and (d) 12 beams simultaneously across the field of view.

As the number of stripes increases, non-homogenous cross-talk can be observed due to non-uniformities in the stripe array emission, resulting in line artefacts in the final image. For instance, a missing stripe in the LED will result in a lower cross-talk in the corresponding raw image and eventually in a dark lines in the multi-beam confocal image, as observed in Fig. 5.7.d.

2.1.2 Haze rejection and sectioning capability

Degradation of confocality was measured using the same fluorescent sheet as before. Images were acquired using a 40X 0.85NA objective with a 200ms camera exposure per pattern position. This optical sectioning measurement was repeated for several line-to-line spacing configurations. Fig. 5.8 shows that cross-talk becomes more and more noticeable as more and more lines are used to illuminate the sample, giving rise to an increased background level, as expected when using a spinning disk microscope with densely packed pinholes or lines.

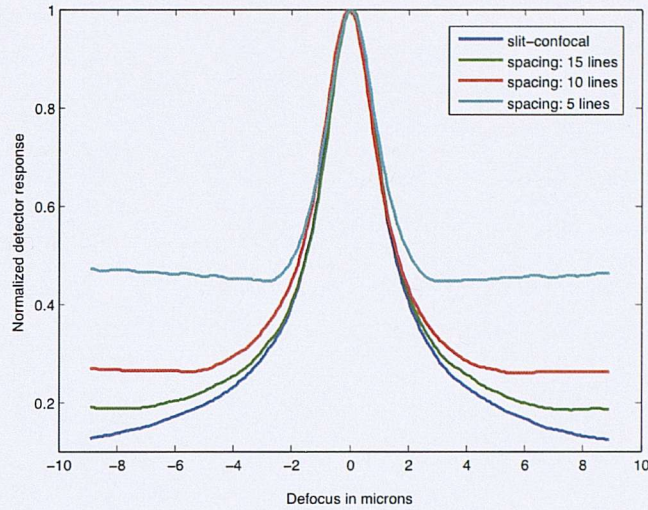


Fig. 5.8. Sectioning strength of the microscope when scanning 1, 8, 12 and 15 beams simultaneously across the object.

3. Method of improving the axial resolution and image contrast in a slit scanning confocal microscope

The concern about removing the out-of-focus offset present in the multi-beam confocal images suggested the following numerical method to improve the axial resolution and image contrast in a slit scanning microscope and eventually obtain a confocal image better than that obtained with a point scanning confocal microscope. Out-of-focus subtraction techniques have already been proven to increase the spatial resolution of various confocal techniques such as Programmable Array Microscopy [61-63] or high fill factor spinning disks [18, 60, 118], where the out-of-focus background is subtracted. Here is investigated the use of what amounts to multiple detection slits to allow the detection of out-of-focus light in order to improve both FWHM response and far from focus signal suppression.

3.1. Principle of haze subtraction when scanning a single beam

Line scanning confocal microscopes using a slit or line illumination source and detector, can provide much faster image acquisition than point scanning microscopes as only one-dimensional scanning is required. However they do not block out-of-focus light travelling along the slit direction, resulting in a full width half maximum (FWHM) sectioning strength approximately 1.5 times larger than that for a point

scanning confocal microscope [5], and a much slower attenuation of signal away from the focal plane.

Consider an optical system consisting of a conventional line-scanning microscope where the detection slit and line detector have been replaced by an array detector, typically a CCD camera. As the beam is scanned across the sample, an image is acquired for each line position. The intensity on the CCD in the j^{th} slit when illuminating the sample at the $i(=j)^{\text{th}}$ stripe position can be written as:

$$I_{i=j}(x) = I_{i=j}^{\text{out}}(x) + I_{i=j}^{\text{in}}(x) \quad (5.10)$$

where x is a given image pixel, and $I_i^{\text{out}}(x)$ is the out-of-focus light and $I_i^{\text{in}}(x)$ the in-focus light when illuminated at the i^{th} illumination position. The latter is detected only by this detector stripe position and attenuates strongly with defocus. However, we note that while the in-focus signal will certainly be different for different illumination positions, the out-of-focus signal will be very similar, particularly when illuminating adjacent stripes. Thus the signal falling in the adjacent detection slit can be written:

$$I_{i=j-1}(x) \approx I_{i=j+1}(x) \approx I_{i=j}^{\text{out}}(x) \quad (5.11)$$

The in-focus intensity component in equation (5.10) attenuates quickly with defocus whereas the out-of-focus intensity does so much more slowly, giving rise to a significant out-of-focus background. However, the signal detected in adjacent stripes can be used as an estimate of the out-of-focus signal and subtracted from the central stripe position stripe position, $j=i$, to obtain an improved confocal image $I_{\text{sec},j}$ by calculating

$$I_{\text{sec},j} = \left[I_{i=j} - \frac{I_{i=j-1} + I_{i=j+1}}{2} \right] \times \text{Slit}_j \approx I_j^{\text{in}} \quad (5.12)$$

3.2. Experimental verification in a conventional slit scanning microscope

3.2.1 Microscope setup

In order to demonstrate the subtraction technique experimentally, the microscope arrangement depicted in Fig. 5.9 was used. For uniformity reasons, illumination was provided by a single stripe-LED, which was projected onto the sample using a 180mm Olympus tube lens and a 20x 0.5NA Olympus dry objective. Fluorescent emission from the sample was imaged by an identical system onto an Orca ER CCD camera

and acquired as a set of 576×64 pixel images each with 100ms exposure. The sample was scanned across the beam using a Marzhauser automated X-Y stage, which released the need for a mirror-based beam scanning system. The illumination was thus stationary with respect to the camera and images were shifted during data processing to produce full two-dimensional images of the sample. The distance between line positions at the sample was set to $0.6\mu\text{m}$, corresponding to 4 optical units at $\lambda=470\text{nm}$.

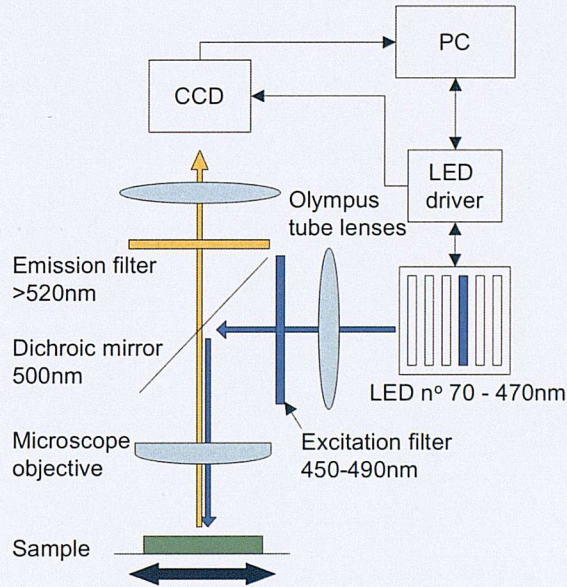


Fig. 5.9. Diagram of the microscope setup used for experiment and numerical simulation

3.2.2 *Data processing*

For convenience, the illumination stripe is assumed to be parallel to the y-axis of the camera sensor. Reconstruction of a confocal image requires several steps:

1. From a reference image $\text{Ref}(x,y)$ obtained by imaging a fluorescent sheet in-focus, a binary slit mask $\text{Slit}(x,y)$ is created using a Canny edge detection function [119]. The Canny method uses two different thresholds (to detect strong and weak edges), and includes the weak edges in the output only if they are connected to strong edges. This method is therefore suited for creating a slit mask as the intensity still varies significantly along the illumination stripe LED. Fig. 5.10 shows the reference image used during the processing and the detected close-loop edge. By selecting the pixels of the area inside the edge to be unity, we create the binary slit mask.

2. Haze is rejected from the raw images using Equation (5.12). For each line position, the three images used in the calculation are shifted relative to each other in software by a number of pixels P corresponding to the actual sampling distance L_β using the expression

$$P = \frac{L_\beta M}{L_{\text{pixel}}}, \quad (5.13)$$

where M is the magnification of the objective and $L_{\text{pixel}} = 6.45 \mu\text{m}$ is the size of a single camera square pixel. A normalized sampling distance of 4 O.U. for instance corresponds to an image shift of 3.7 pixels with the ORCA camera.

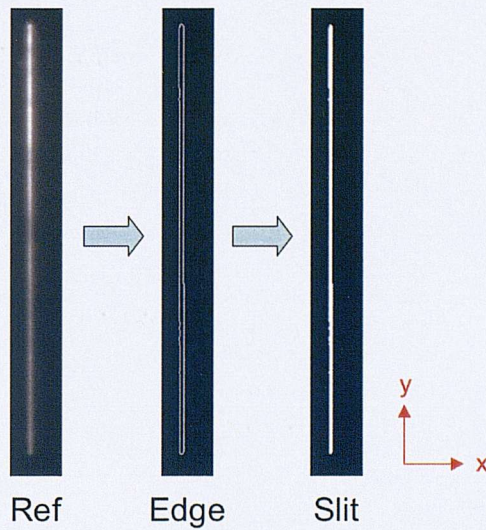


Fig. 5.10. Binary slit using a Canny edge detection filter

3. Calculated images are converted into columns of pixels and normalized by the number of pixels in the slit width.

The i^{th} column of the final improved confocal image $\text{IMP}(x,y)$ can be calculated using the equation:

$$\text{IMP}(x = i, y) = \frac{\sum_x \left[I_i(x, y) - \frac{1}{2} (I_{i-1}(x - P, y) + I_{i+1}(x + P, y)) \right] \times \text{Slit}(x, y)}{\text{Norm}(y)} \quad (5.14)$$

where $\text{Norm}(y)$ represents the varying slit width

$$Norm(y) = \sum_x Slit(x,y) \quad (5.15)$$

Similarly, a conventional confocal slit scanning image $CONF(x,y)$ can be reconstructed which i^{th} column is described by the expression:

$$CONF(x=i,y) = \frac{\sum_x I_i(x,y) \times Slit(x,y)}{Norm(y)} \quad (5.16)$$

Finally a widefield image $WF(x,y)$ can be calculated by summing the shifted raw images together:

$$WF(x,y) = \sum_i [I_i(x+iP,y)] \quad (5.17)$$

3.2.3 Correction for illumination non-uniformity

Because only the light coming from the in-focus object remains in the improved image $IMP(x,y)$, this image can be corrected for non-uniformities within the single stripe illumination without introducing additional artefacts. Then a new normalization can be used.

$$Norm(y) = \frac{\sum_x Ref(x,y) \times Slit(x,y)}{Max\left(\sum_x Ref(x,y) \times Slit(x,y)\right)} \quad (5.18)$$

This normalization factor corrects for the varying slit width and the variations of the illumination in the direction y parallel to the slit. However this correction cannot be implemented on the normal confocal image $CONF(x,y)$, as a significant amount of out-of-focus light is still present. Because the information about where this out-of-focus light originates has been lost with defocus, applying the above correction results in improving the image of the in-focus object at the expense of introducing substantial artefacts in the out-of-focus part of the image.

3.2.4 Images

Several samples were imaged and processed using the above technique. Fig. 5.11 shows several fluorescence images of a cluster of stained pollen grains. Exposure time was 100ms per line position with the single stripe LED driven at 3mA. 108 line positions were acquired with a sampling distance of $0.6\mu m$, which corresponds to

about 4 optical units using a 20X 0.5NA objective at $\lambda=470\text{nm}$. Fig. 5.11(b)-(c) illustrates how haze rejection is improved by subtraction followed by correction for illumination non-uniformities. It is clear that the out-of-focus blur remaining in the conventional slit-scanning image has been efficiently removed in the improved image.

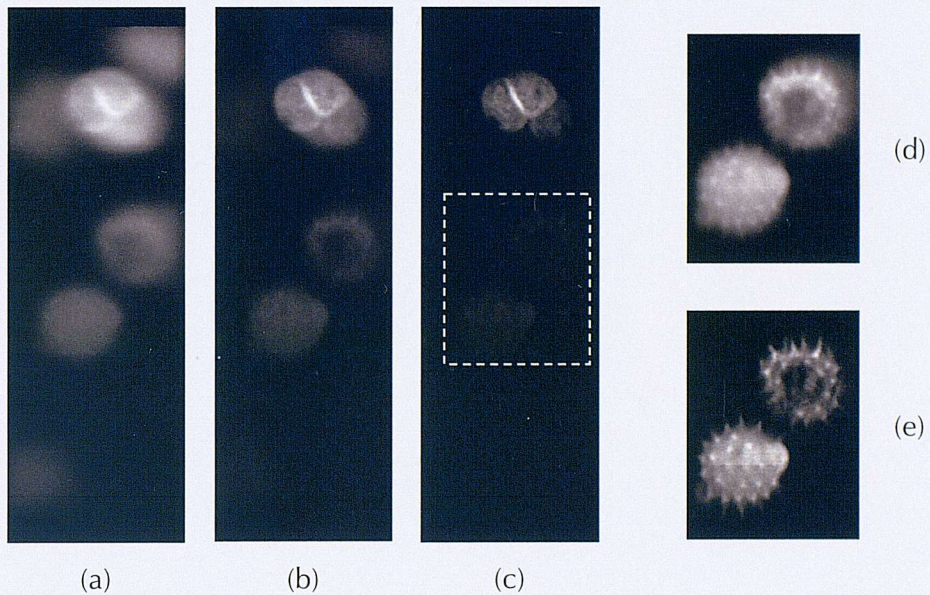


Fig. 5.11. Fluorescence images of a cluster of pollen grains imaged with a 20X 0.5NA lens. Scale bar is 20 microns. (a) Reconstructed widefield image. (b) Conventional slit confocal image. (c) Improved slit confocal image. (d)-(e) Auto-scaled zoom of the selected pollen grains. Images (d) and (e) are taken from images (b) and (c), respectively.

Fig. 5.11(d)-(e) show enlarged auto-scaled images of the faint in-focus cluster highlighted in the previous image. Details such as pollen grain spikes or inner grain structures are clearly best resolved in the improved image than in the conventional one.

Human Embryonic Kidney (HEK293) cells were also imaged using the same technique (Fig. 5.12). Cells were stained with a dye (di-4-ANEPPDHQ) that incorporates itself into the cell lipid bilayers where it becomes fluorescent [120-122]. The sampling distance was $0.4\mu\text{m}$, which again corresponds to 4 optical units using a 40X 0.75NA objective and the beam was scanned over 200 positions. Exposure time was 300ms per position, which resulted in an overall acquisition time of 60 seconds. Fluorescent structures observed in Fig. 5.12 are mainly intra-cellular membranes, such as Golgi apparatus or mitochondria membranes. Due to the lack of inner membranes,

the nucleus does not fluoresce and appears as a dark round object inside the cells surrounded by bright structures.

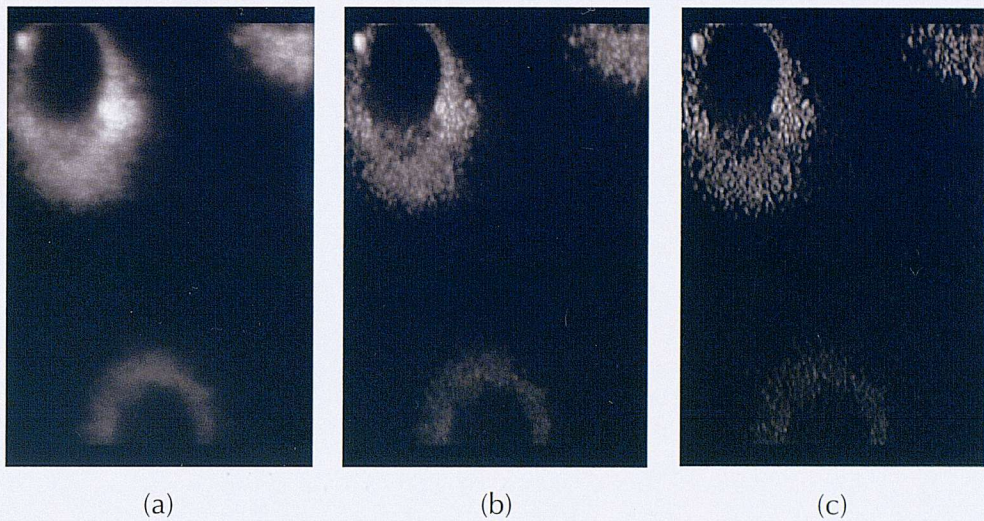


Fig. 5.12. Fluorescence images of stained HEK cells acquired using a 40X 0.75NA objective: (a) Reconstructed widefield image. (b) Conventional slit scanning image. (c) Enhanced resolution image

3.3. Simple microscope design for enhanced axial resolution

Instead of scanning the sample, modern line-scanning confocal microscopes usually scan the illumination beam across the field of view. Scanning the beam has advantages in terms of speed and is achieved using a fast scanning mirror [55] or an acousto-optic modulator (AOM) [54]. The light coming back from the sample is de-scanned by the scanning unit and passed through a detection slit, which blocks the out-of-focus light and produces the confocal effect. The in-focus light is then imaged onto a line detector.

By replacing the current detection slit and line-detector by a 3-column array detector, the subtraction technique could be easily implemented (Fig. 5.13). The middle pixel column would capture the light coming mainly from the in-focus region whereas the side columns would capture the out-of-focus light to be subtracted from the surrounding beam positions. Haze subtraction could take place in real time and could be implemented directly in the frame-grabber controller so that only true confocal images would be transferred to the host computer. Tri-linear detectors already exist and are commonly used in colour photocopiers to record a colour image

of a scanned document but spacing between pixel columns is currently of the order of tens of microns, which results in a very low fill-factor.

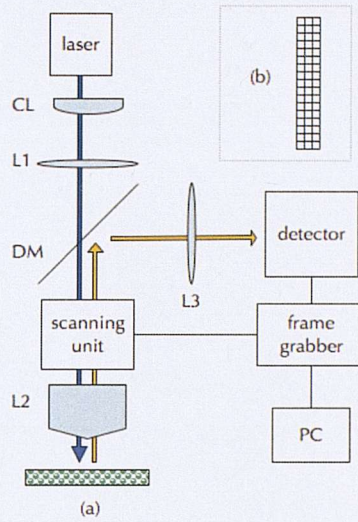


Fig. 5.13. Modified line-scanning microscope for enhanced axial resolution. (a) Microscope setup. L's are convex lenses, CL is a cylindrical lens and DM is a dichroic mirror. (b) Three-column CCD detector.

4. Solid state microscope with improved optical sectioning

The micro-stripe LED was used to scan the line illumination pattern onto the sample rather than scanning the specimen using an automated stage. Both single line and multiple line illumination patterns were used and the background subtraction technique was implemented. However, due to array non-uniformities the processing used for subtraction had to be modified to deal with broken stripes, non-uniform cross talk and stripe-to-stripe differences in emission power.

4.1. Single line scanning microscope

4.1.1 *Processing*

The microscope setup remains the same as for the conventional slit microscope (Fig. 5.1) described in the first section. The imaging process starts with the acquisition of $N=120$ references images, which are used for generating the 120 confocal slits, as previously described in the first section of this chapter. Widefield images of the specimen are then captured under single stripe illuminations. The out-of-focus light to be subtracted could not be estimated by looking at the light detected while illuminating the specimen with the adjacent stripes, as described in the previous

section, because of severe emission non-uniformities across the LED array. Instead estimating this out-of-focus light by looking at the light captured by the neighbouring detection slits in the same image allows overcoming most of the non-uniformity issues. This is illustrated in Fig. 5.14 where the out-of-focus contributions are measured directly from the detection slits $i-1$ and $i+1$.

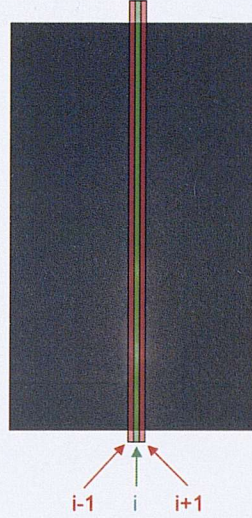


Fig. 5.14. In-focus and out-of-focus contribution in a pollen grain image under single stripe illumination

Subtraction of the out-of-focus contribution can then be calculated using

$$Sec(x=i, y) = \frac{Conf(x=i, y) - \frac{1}{2} \left[\frac{\sum_x I_i(x, y) slit_{i-1}(x, y)}{\sum_x slit_{i-1}(x, y)} + \frac{\sum_x I_i(x, y) slit_{i+1}(x, y)}{\sum_x slit_{i+1}(x, y)} \right]}{Norm_i(y)} \quad (5.19)$$

where $Conf$ is the conventional slit confocal image given by Equation (5.16) and $Norm$ is the normalisation function given by

$$Norm_i(y) = \frac{\sum_x Ref_i(x, y) \times Slit_i(x, y)}{Max \left(\sum_x Ref_i(x, y) \times Slit_i(x, y) \right)} \times \frac{\sum_x \sum_y Ref_i(x, y) \times Slit_i(x, y)}{Max_{N=120} \left(\sum_x \sum_y Ref_i(x, y) \times Slit_i(x, y) \right)} \quad (5.20)$$

In the normalisation function the first term corrects for the emission non-uniformity inside a single emitter, whereas the second term corrects for the stripe-to-stripe mismatch in emission power. It should be noted that the normalisation is

applied to each slit after background subtraction to ensure correction of only the in-focus component without adding additional artefacts.

4.1.2 Results

Images of pollen grain cluster generating a significant amount of out-of-focus signal were acquired using a 40x 0.75NA dry objective and processed using this method. The specimen was illuminated for 100ms per stripe. Fig. 5.15 shows the reconstructed widefield image, the conventional slit confocal image and the subtraction image after non-uniformity correction.

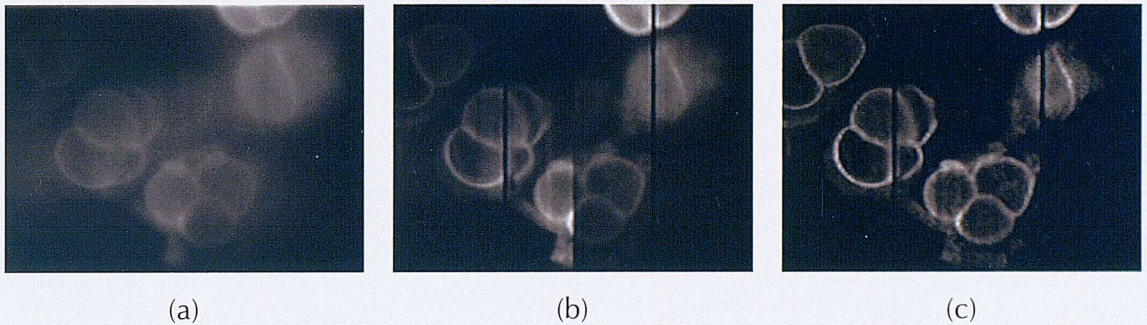


Fig. 5.15. Pollen grain cluster imaged using a 40x objective: (a) Reconstructed widefield image, (b) Conventional slit confocal image and (c) Subtraction confocal image after artefact correction

From Fig. 5.15 (c) it is clear that most of the background light still present in the confocal image has been subtracted and that the image is well corrected, apart from two broken LEDs that result in black stripes in the improved image.

4.2. Multiple line scanning microscope

Similar processing can be readily applied to images under multiple stripe pattern illuminations. By subtracting the adjacent out-of-focus contributions and by processing and correcting each stripe independently, non-homogeneous cross-talk that was described earlier can be suppressed. After reprocessing, confocal images are dramatically improved as shown in Fig. 5.16. It is clear that haze and cross-talk offset are completely removed by subtraction and that line artefacts are suppressed in the improved images. It can be noted that improved images look very similar whatever line pattern is used, whereas the confocal images degrade considerably as the number of lines is increased.

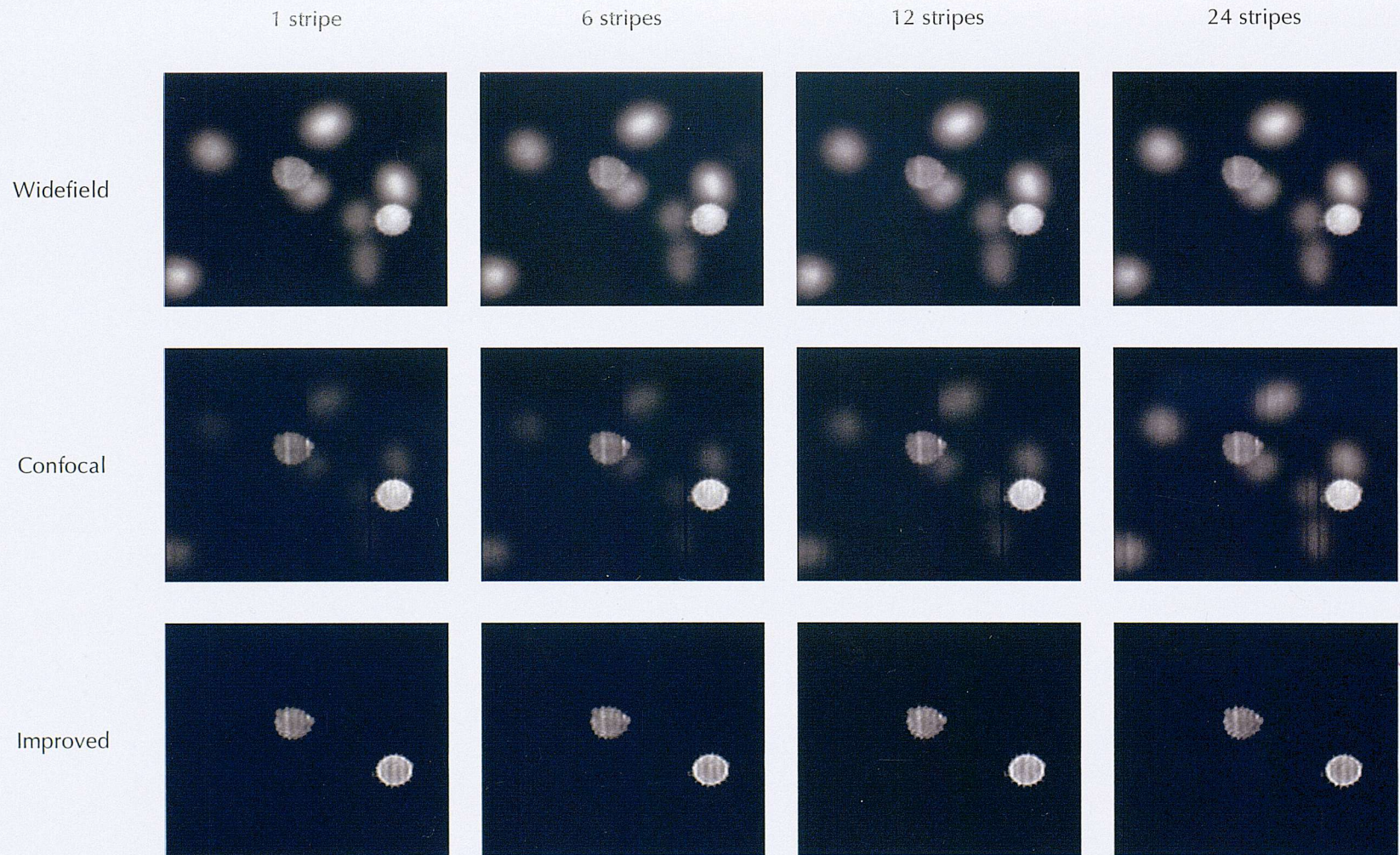


Fig. 5.16. 20X Widefield, Confocal and Improved fluorescence images of a pollen grain cluster under 1, 6, 12 and 24 stripe illuminations

4.3. Advantages of the solid-state microscope with improved sectioning

Speed and flexibility are the main advantages of the solid-state system. The microscope user can increase the confocal frame rate by changing the number of stripes in the illumination pattern and still obtain an artefact-free confocal image with improved optical sectioning. On the other hand, signal to noise can be improved by reducing the fill-factor of the illumination pattern when imaging a highly scattering object.

5. Summary

A solid-state line scanning confocal microscope with no moving-parts was presented and characterised. Its optical sectioning ability was successfully compared with the theory. A new method to reject the residual out-of-focus light in the line-scanning sectioned image is introduced that allows for dramatic axial resolution improvement over the conventional line scanning system. Because the micro-stripe LED device makes it possible to electronically select the illumination line pattern, multiple lines can be scanned in parallel at the expense of an intensity offset. The same subtraction method to reject out-of-focus light in single line-scanning systems can be applied to multi-slit images and gives the microscope user the opportunity to match its confocal system to the specimen to be imaged.

Chapter 6 Theory of optical sectioning by out-of-focus background subtraction

In the previous chapter, a subtraction method was suggested to remove the out-of-focus background in a line scanning microscope and experiments demonstrated a dramatic improvement of the optical sectioning and image contrast under both single line and multi-line illuminations. This chapter describes the theory of optical sectioning improvement by out-of-focus background subtraction in a line-scanning microscope. Axial resolution obtained is 2.2 times better than the slit confocal resolution and out-of-focus suppression is calculated to attenuate with defocus at almost an order of magnitude faster than in the point confocal case. The theory is then extended to multiple-line illumination patterns and is shown to converge towards the structured illumination theory in the limit when illuminating the specimen with a high fill-factor grid pattern. Finally, the theory is applied to the analysis of the sectioning performance of a double-sided disk microscope, and a new design of the disk is proposed to achieve light-efficient confocal microscopy with superior optical sectioning at very high frame rate.

1. Improving optical sectioning in a slit scanning microscope

1.1. Sectioning capability

The performance of the single-line subtraction microscope described in chapter 5 is best appreciated by considering an object consisting of an infinitely thin fluorescent sheet lying in a plane orthogonal to the optic axis as this represents the most difficult type of object in terms of out-of-focus haze. Assuming finite width, infinite length illumination and detection slits aligned parallel to the y-axis and incoherent illumination, the response to defocus of a simple slit-scanning microscope can be written as:

$$I(u, \beta) = C \int_{-2}^{+2} T^2(s_x, 0, u) \text{sinc}\left(\frac{ps}{2\pi}\right) \text{sinc}\left(\frac{qs}{2\pi}\right) \exp(-is\beta) ds_x \quad (6.1)$$

where the lateral shift between illumination and detection slits is given by the distance β , and their widths are given by p and q respectively, all measured in normalised lateral optical units.

$$\beta = \frac{2\pi n}{\lambda} x \sin(\alpha) \quad (6.2)$$

$T(s, u) = T(s_x, s_y, u)$ is again the incoherent OTF of the microscope, which is approximated by Stokseth at low NA. Introducing Equation (6.1) into Equation (5.13), the defocus response of the subtraction microscope can be written as:

$$\begin{aligned} I_{sub}(u) &= \left[I(u, 0) - \frac{I(u, \beta) + I(u, -\beta)}{2} \right] \\ &= C_s \int_{-\infty}^{+\infty} T^2(s, u) \text{sinc}\left(\frac{ps}{2\pi}\right) \text{sinc}\left(\frac{qs}{2\pi}\right) [1 - \cos(s\beta)] ds \end{aligned} \quad (6.3)$$

Similarly the simple slit scanning confocal response, $I_{slit}(u)$, and the simple point scanning response, $I_{point}(u)$, can be written as:

$$I_{slit}(u) = C_s \int_0^{+\infty} T^2(s, u) \text{sinc}\left(\frac{ps}{2}\right) \text{sinc}\left(\frac{qs}{2}\right) s ds \quad (6.4)$$

$$I_{point}(u) = C_p \int_0^{+\infty} T^2(s, u) \text{jinc}\left(\frac{ps}{2}\right) \text{jinc}\left(\frac{qs}{2}\right) s ds \quad (6.5)$$

where $\text{jinc}(x) = J_1(x)/x$ and p and q are also the diameters of the illumination and detection pinholes in the case of the point scanning microscope.

A simple analysis of the asymptotic form of equations (6.3), (6.4) and (6.5) can be carried out to describe the microscope responses at large defocus. Noting that $T(s, u)$ has significant values only at low spatial frequency for large defocus, these equations can be simplified to:

$$\begin{aligned} I_{slit}(u) &\propto \int_0^2 \left(\frac{J_1(us)}{us} \right)^2 ds = o(1/u) \\ I_{point}(u) &\propto \int_0^2 \left(\frac{J_1(us)}{us} \right)^2 s ds = o(1/u^2) \\ I_{sub}(u) &\propto \int_0^2 \left(\frac{J_1(us)}{us} \right)^2 s^2 ds = o(\ln(u)/u^3) \end{aligned} \quad (6.6)$$

which suggests the superior performance of the subtraction microscope in rejecting the haze compared with the point scanning microscope.

1.2. Simulations

Results of simulations for a diffraction limited illumination and detection ($p=q=0$) are shown in Fig. 6.1. As observed in Fig. 6.1 (a)-(c), where the sampling distance β is varied from 4 to 20 O.U., haze rejection is more efficient for small sampling distances, although this goes at the expense of rejecting some of the in-focus signal. It can be observed in Fig. 6.1 (d) that the subtraction microscope becomes marginally better than the point scanning one for $\beta \leq 4$ O.U.

The sectioning strength improvement and the decrease of the signal strength were quantified for several sampling distances (Fig. 6.1 (e)-(f)). Sectioning can be improved at best 1.6 fold in a diffraction-limited system compared to the conventional slit-scanning microscope. However, the remaining in focus signal rapidly drops to zero for sampling distance smaller than 5 O.U. The sampling distance $\beta=4$ O.U. seems a good trade-off as it results in a sectioning strength similar with a point microscope while still capturing 84% of the in-focus signal of the line-scanning microscope.

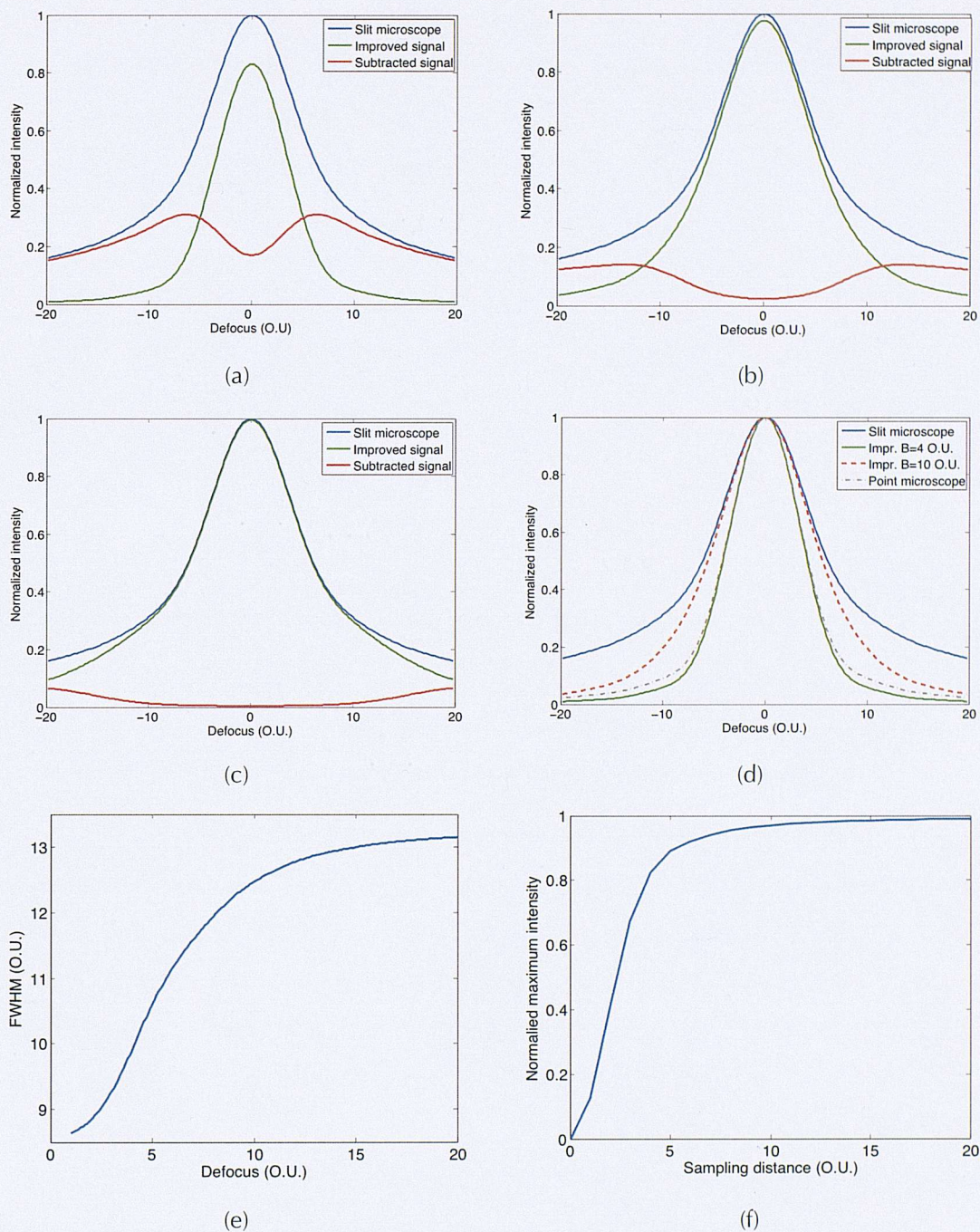


Fig. 6.1. Simulation results in the case of a diffraction limited illumination and detection slits ($p=q=0$). (a) Defocus response for the conventional slit microscope (blue) and improved microscope (green), and subtracted signal (red) for $\beta=4$ O.U. (b)-(c) Same with $\beta=10$ and 20 O.U., respectively. (d) Normalized detector responses for a slit microscope (blue), Improved microscope for $\beta=4$ and 10 O.U. (green and red) and diffraction limited point scanning microscope. (e) Sectioning strength improvement and (f) in-focus signal strength as the sampling distance varies.

The performance of the three systems far from focus is illustrated in the log-log plots shown in Fig. 6.2, where much improved rejection of out-of-focus light can be seen with the subtraction technique. Here, slopes of the residual signal on the log-log plots were estimated as -1.0, -2.0 and -2.9 for the slit, point and subtraction microscope, respectively, which agrees with the asymptotic forms of the equations.

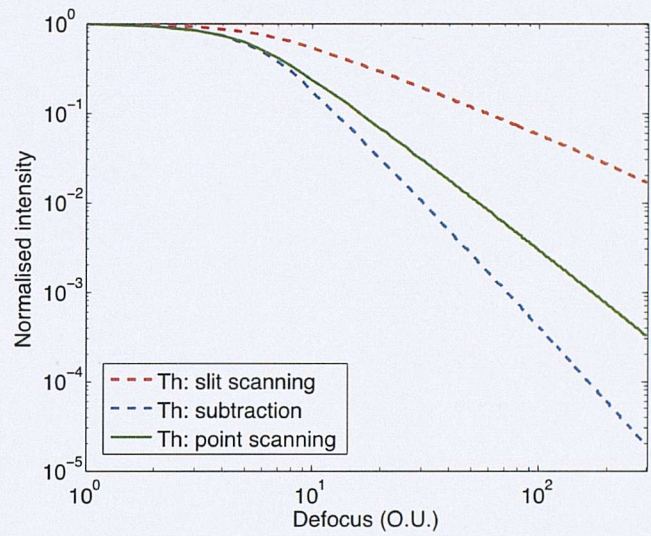


Fig. 6.2. Simulated residual intensity showing improved background suppression of the subtracted signal at large defocus.

Finally, Fig. 6.3 shows the simulated normalised response with defocus for a slit-scanning microscope, a point scanning microscope and the subtraction microscope modelled for the same parameters as the experimental setup described earlier in chapter 5. Also shown are experimental measurements made for the slit scanning and the subtraction case using a thin fluorescent sheet imaged with a 20X 0.5 dry objective. Illumination and detection slits were chosen to be 17 and 34 μm wide, which correspond to 5.5 and 11 O.U. in the focal plane, respectively and the sampling distance was here $\beta = 0.6\mu\text{m}$ (or $\beta = 4$ O.U.). Simulated and experimental results agree well and the FWHM of the simulations were found to be 7.2 μm , 3.4 μm and 3.3 μm , respectively, implying that the sectioning capability of the subtraction system is 2.2 times better than the slit microscope and marginally improved over the point scanning microscope.

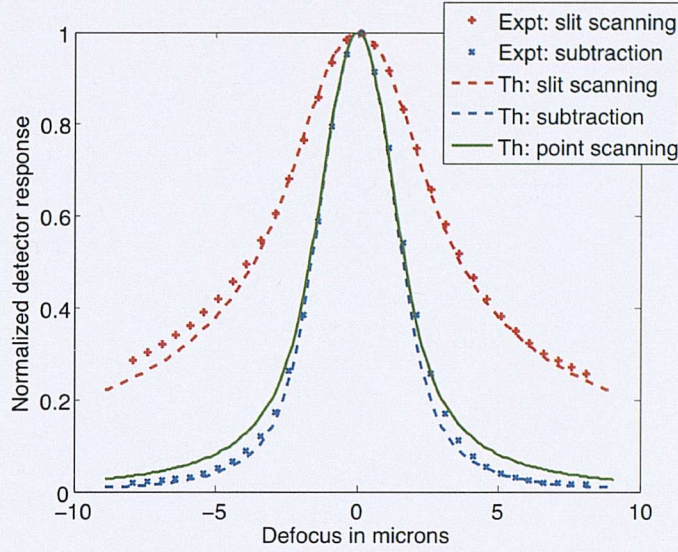


Fig. 6.3. Experimental and numerical simulation of the response of the microscope to a thin fluorescent sheet scanned through the focal plane. Numerical simulation of the performance of a point scanning microscope is also shown for comparison.

1.3. Subtraction microscope in presence of photon noise

In any subtraction technique the noise is limited by the noise in the acquired images. Therefore, the noise in the subtraction sectioned image is roughly equivalent to that of the slit-scanning microscope. This will result in a noise floor declining as $o(u^{-1/2})$ in this particular subtraction microscope configuration. Assuming normally distributed measurements ($\Delta I = \sqrt{I}$) and using Equation (5.13), the noise floor in the subtraction microscope can be described as:

$$\begin{aligned}
 \Delta I_{sub}(u, \beta) &= \sqrt{(\Delta I_{slit}(u, 0))^2 + \frac{(\Delta I_{slit}(u, \beta))^2}{2}} \\
 &= \sqrt{I_{slit}(u, 0) + \frac{I_{slit}(u, \beta)}{2}} \\
 &= o(1/\sqrt{u}) \quad \text{at large defocus}
 \end{aligned} \tag{6.7}$$

In order to compare the noise of the line, point and subtraction systems at large defocus, the theoretical sectioning curves shown in Fig. 6.3 are normalised by the sample exposure. However, because the detection geometry is different in the slit and point cases, the detection efficiencies for a given exposure have to be calculated first. Assuming that the microscope objective captures N fluorescence photons emitted by

an in-focus fluorescent object, the number of photons that pass through the detection slit can be calculated as

$$N_{det,slit} = Eff_{slit} N \quad (6.8)$$

where Eff_{slit} is the detection efficiency of the slit microscope. Rewriting Equation (6.4) to maintain the correct normalisation, the detection efficiency for the slit microscope can be written:

$$Eff_{slit} = \frac{q}{\pi} \int_0^2 T^2(s,0) \text{sinc}\left(\frac{ps}{2\pi}\right) \text{sinc}\left(\frac{qs}{2\pi}\right) ds \quad (6.9)$$

It can be checked that

$$Eff_{slit} = \begin{cases} 1 & q \rightarrow +\infty \\ 0 & q = 0 \end{cases} \quad (6.10)$$

which corresponds to the widefield limit when a infinite detector is used ($q \rightarrow +\infty$) and to the case when the detection slit is closed. Similarly, the detection efficiencies of the point and subtraction systems can be calculated using:

$$\begin{aligned} Eff_{sub} &= \frac{q}{\pi} \int_0^2 T^2(s,0) \text{sinc}\left(\frac{ps}{2\pi}\right) \text{sinc}\left(\frac{qs}{2\pi}\right) [1 - \cos(\beta s)] ds \\ Eff_{point} &= \frac{q^2}{2} \int_0^2 T^2(s,0) \text{jinc}\left(\frac{ps}{2}\right) \text{jinc}\left(\frac{qs}{2}\right) s ds \end{aligned} \quad (6.11)$$

If the sampling distance β is chosen to be 4 O.U. and illumination and detection slits are chosen to be 17 and 34 μm wide, the detection efficiencies can be calculated to be 0.74, 0.58 and 0.28 for the slit, point and subtraction systems, respectively.

Simulations of the three systems are shown in Fig. 6.4 for several numbers of photons gaining admittance through the microscope objective. The red, green and blue curves represent the response to defocus of the slit, point and subtraction systems, respectively. The dotted curves represent the corresponding photon noise levels.

For each microscope, the real response to defocus corresponds to the solid line until the noise level overcomes the remaining signal. The point microscope has a noise floor declining as $o(u^{-1})$ at large defocus. As described in Equation (6.7) the noise floor of the subtraction system is higher than the slit one for small defocus, and converges rapidly towards the noise floor of the line system when defocus increases. The drawback of the subtraction technique compared to the point technique is that

the measurement becomes rapidly noise limited for a low photon count. Assuming a sampling distance of $\beta=4$ O.U., the defocus positions at which the subtraction microscope becomes limited by the shot noise was found to be 9, 15 and 26 O.U. for 100, 1000 and 10,000 captured photons, respectively.

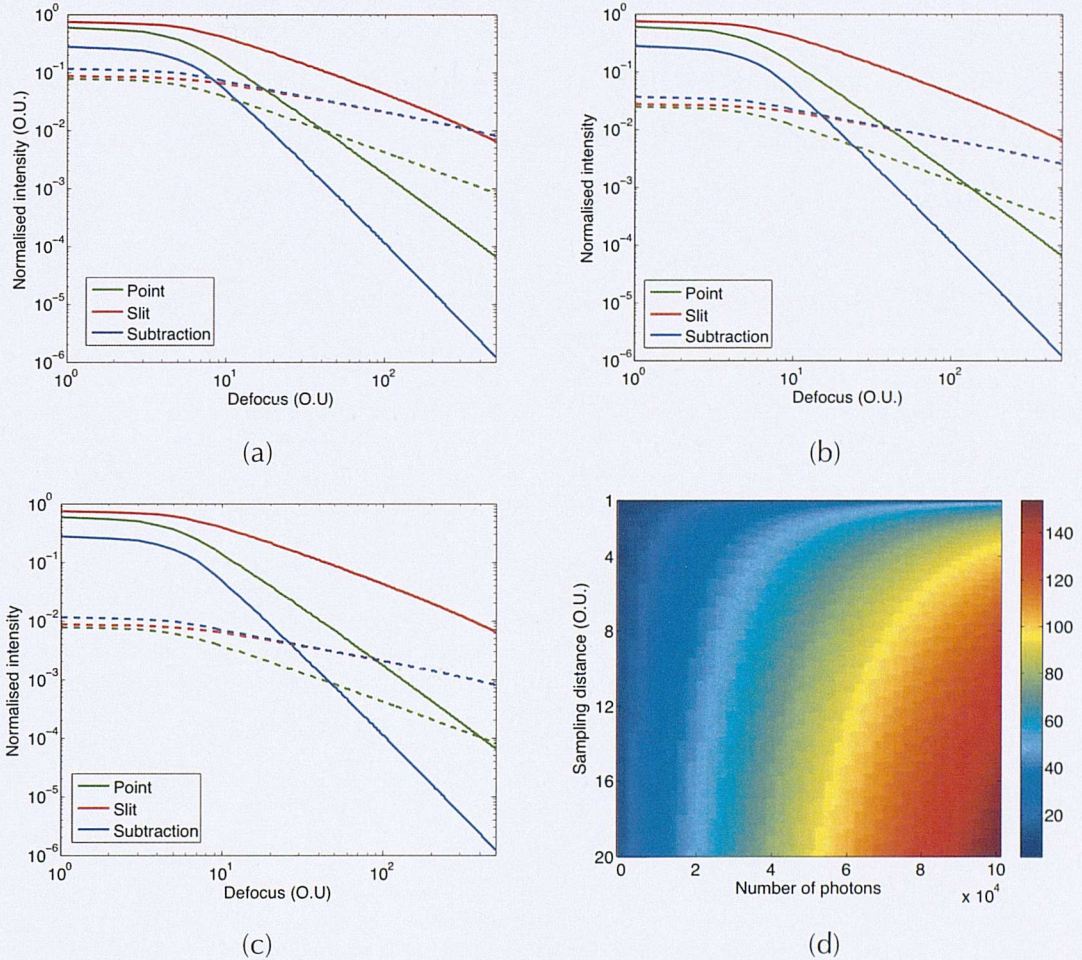


Fig. 6.4. Point scanning (green), slit scanning (red) and subtraction (blue) in presence of photons noise (dotted curves) for 100 (a), 1000 (b) and 10,000 (c) captured photons. All the curves were normalised by the number of captured photons. Sampling distance is here 4 O.U. for the subtraction microscope. (d) Axial position where the subtraction technique becomes noise limited for several sampling distance and an increasing numbers of photons. The colour bar represents the axial position in O.U.

Fig. 6.4 (d) shows a map of the axial positions at which the noise floor overcomes the subtracted signal for different sampling distance and for several numbers of photons. As expected the subtraction microscope does not perform well at low photon counts or small sampling distances, where most of the in-focus-signal is subtracted but the noise still adds up.

It is of interest to study how the noise floor varies when changing the sampling distance β . From Equation (6.7), it is clear that

$$\begin{aligned} \beta \rightarrow \infty \quad \Delta I_{sub}(u) &= \sqrt{I_{slit}(u,0)} \\ \beta = 0 \quad \Delta I_{sub}(u) &= \sqrt{\frac{3}{2} I_{slit}(u,0)} \end{aligned} \quad (6.12)$$

which suggests that the relative noise only weakly depends on β . However the uncertainty strongly depends on the number of collected photons, as does the axial position at which the noise floor crosses the PSCM curve. Finally Fig. 6.5 shows the defocus position at which the sectioning ability of the subtraction microscope becomes worse than the point scanning system due to the noise floor. The point scanning system performs better for very low photon counts. However, the difference at large defocus becomes hardly noticeable as the number of collected photons increases.

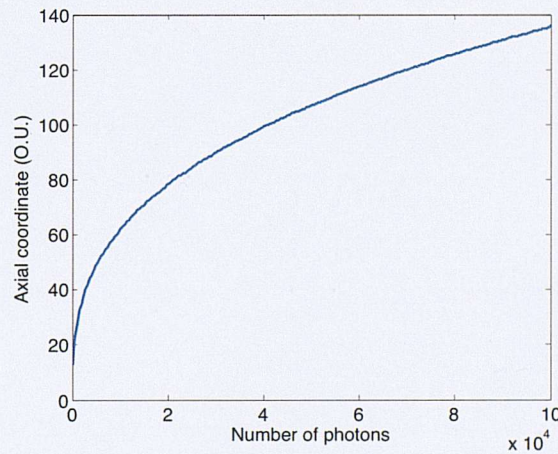


Fig. 6.5. Defocus position at which the noise floor overcomes the point scanning microscope signal. Here $\beta=4$ O.U.

2. Axial resolution enhancement when scanning multiple lines

It was experimentally shown in the previous chapter (Fig. 5.8) that confocality is seriously degraded due to detection cross-talk as more and more beams are scanned simultaneously on the sample, using for instance a micro-structured LED as the illumination source. As more and more lines are scanned in parallel, haze starts increasing and results in an intensity offset that does not attenuate with defocus. This offset can be estimated by looking at the intensity falling in the surrounding area outside the detection slit and can be subtracted from the raw image to obtain a true confocal image. As only in focus light remains, it was experimentally shown that corrections could be applied to remove line artefacts resulting from non-homogeneous line illumination.

2.1. Multi-stripe subtraction system

2.1.1 Principles

In order to obtain a true confocal image, the same imaging theory can be described when applying the subtraction method to the set of multi-line images. Assuming a N-line illumination pattern having a normalised period $\delta = (2\pi n/\lambda)D\sin(\alpha)$, where D is the physical grid period, Equation (6.13) describes the response to defocus of the subtraction system:

$$I_{\text{Msub}}(u, \beta, \delta) = \sum_{k=-N}^N \left[I(u, k\delta) - \frac{I(u, k\delta - \beta) + I(u, k\delta + \beta)}{2} \right] \quad (6.13)$$

When imaging a fluorescent planar object, introducing Equation (6.1) into Equation (6.13) gives

$$I_{\text{Msub}}(u, \beta, \delta) = C_s p q \int_{-\infty}^{+\infty} T^2(s, u) [1 - \cos(s\beta)] \text{sinc}\left(\frac{ps}{2\pi}\right) \text{sinc}\left(\frac{qs}{2\pi}\right) \left[\sum_{k=-N}^N e^{ik\delta s} \right] ds \quad (6.14)$$

In the limit when $N \rightarrow +\infty$ and defining the grid normalised frequency $\nu = 2\pi/\delta$, this can be written as:

$$\begin{aligned}
I_{Msub}(u, \beta, \delta) &= \frac{4\pi^2}{\delta^2} pq \int_{-\infty}^{+\infty} T^2(s, u) [1 - \cos(\beta s)] \text{sinc}\left(\frac{ps}{2\pi}\right) \text{sinc}\left(\frac{qs}{2\pi}\right) \left[\sum_{k=-\infty}^{\infty} \delta\left(s - k \frac{2\pi}{\delta}\right) \right] ds \\
&= v^2 pq \sum_{0 \leq k \leq \frac{2}{v}} T^2(kv, u) [1 - \cos(k\beta v)] \text{sinc}\left(\frac{kp v}{2\pi}\right) \text{sinc}\left(\frac{kq v}{2\pi}\right)
\end{aligned}
\tag{6.15}$$

It can be noted that the response to defocus of the structured illumination technique is found when using a grid type illumination ($\delta = 2\beta$). In this case the objective lens only captures odd spatial harmonics. When using a sufficiently fine square grid ($1 < v \leq 2$), only the first diffraction order is imaged, which corresponds to the structured illumination case using a single frequency grid. However, the processing algorithm is different and modulated noise patterns such as described in chapter 3 for the structured illumination system should not be present in the sectioned image. Finally it can be seen that the maximum sectioning strength corresponds to $\delta = 2\beta = 2\pi$, which is similar to the structured illumination microscope when the grid frequency is perfectly matched with the characteristics of the objective lens ($\tilde{\xi} = 1$).

2.2. Simulations

Equation (6.15) was used to simulate the response to defocus of a multi-slit subtraction microscope for different slit-to-slit arrangements. Fig. 6.6 shows the multi-slit (a) and the resulting subtraction signal (b) in case of very narrow illumination and detection slits ($p=q=0$). In these simulations $\beta=\pi$ so that the optimal setup for structured illumination microscope is found if $\delta=2\pi$. As the period of the line pattern is increased, out-of-focus cross talk starts decreasing while the sectioning strength degrades, as seen in Fig. 6.6 (b). For $\delta > 7\pi$ the response to defocus of the multi-line system becomes very similar to the single line response after subtraction. The axial response of the point, line, subtraction and multi-line microscopes are shown in Fig. 6.6 (c) and (d). On the log-log plot, the estimated slope at large defocus of the residual multi-beam signal was estimated as -3, which suggests the superior out-of-focus rejection capability of this technique. This can be again proven using an asymptotic

approach expansion for the Bessel function. Indeed for a sufficiently fine grid ($1 < v \leq 2$), Equation (6.15) describes the response to defocus to vary as

$$I_{Msub}(u, \beta, \delta) \propto T^2(v, u) \propto \left(\frac{J_1(us)}{us} \right)^2 \quad \text{at large defocus} \quad (6.16)$$

which declines as $o(u^{-3})$ at large defocus.

The diffraction-limited case represents the best sectioning that can possibly be achieved, but this goes at the expense of a very low light level as the detection slit is narrow. More realistically, illumination and detection slit widths were chosen to be 17 and 34 μm , respectively, and $\beta = 34 \mu m$ which is the stripe LED centre-to-centre spacing. Thus these simulations represent what can be expected when using the micro-stripe LED as an illumination source.

First, simulated multi-stripe signals for different line patterns (Fig. 6.7 (a)) are consistent with the experimental sectioning curves measured in chapter 5. As more and more stripes are scanned simultaneously on the sample, an intensity offset appears that does not attenuate with defocus.

The response to defocus of the multi-line system when varying the sampling distance from $\beta = W_{det} = 17 \mu m$ to $\beta = 25 \times W_{det}$ is shown in Fig. 6.7 (b)-(c). The improvement in the sectioning of the multi-line subtraction microscope over the single line system becomes noticeable when β becomes less than 5 times the width of the detections slit.

Finally, it can be observed in Fig. 6.7 (d) that the sectioning strength after haze subtraction can be much improved by driving multiple stripes in parallel. The FWHM of the simulated responses were found to be 18 O.U., 11.2 O.U., 10.4 O.U. and 8.8 O.U. for the slit, single slit subtraction, point and multi-subtraction system with $\delta = 2W_{det}$, respectively, implying that the sectioning capability of the multi-subtraction system can be up to twice as good as the slit microscope and 1.2 fold improved over the point scanning microscope.

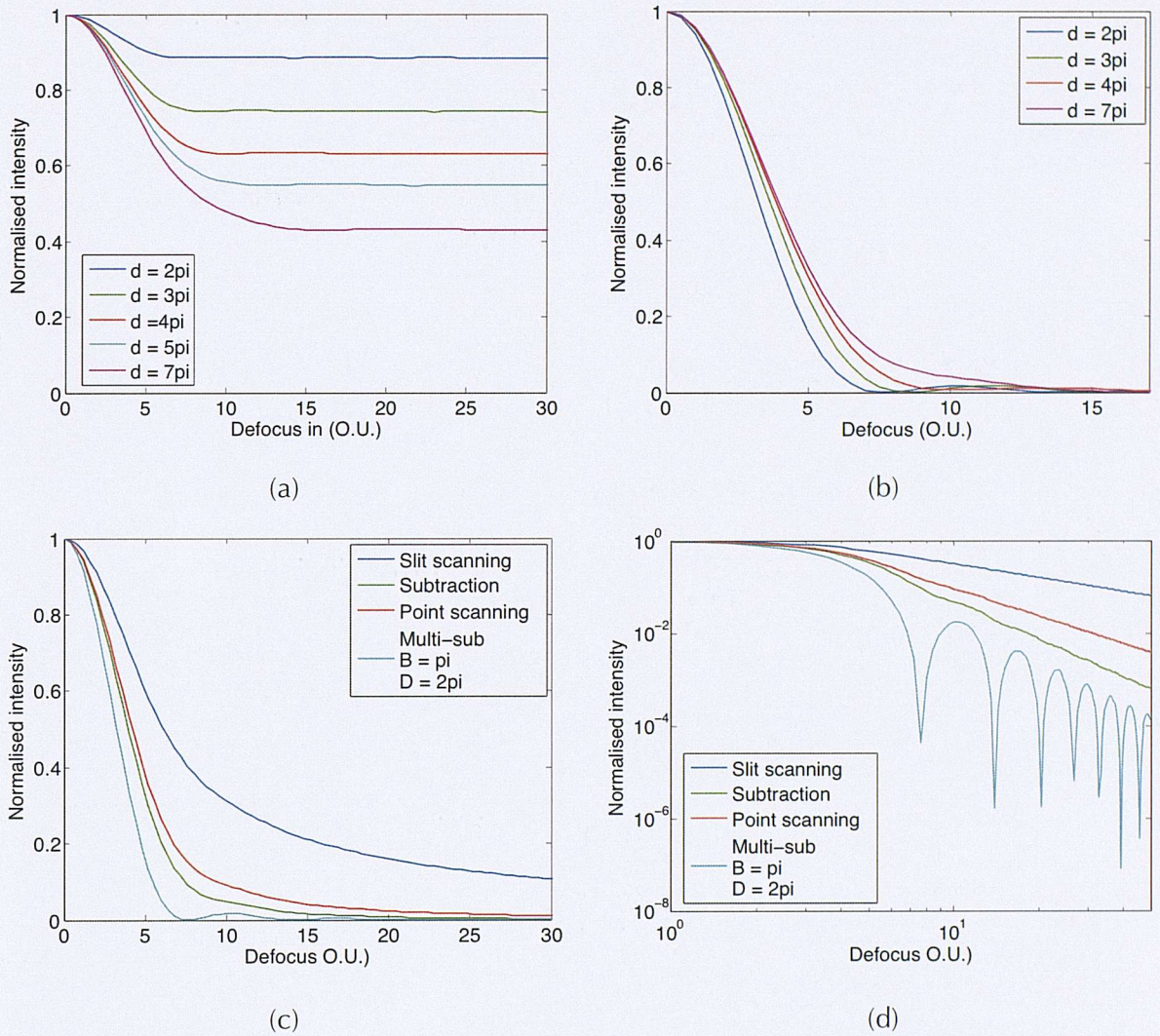


Fig. 6.6. Simulation of a multi-line diffraction limited subtraction system. Here $p=q=0$ and $\beta=\pi$ O.U. (a) Detector signal before background subtraction for different values of δ (d in the figures). (b) Normalised signal after subtraction for the same values of δ . (c) Slit-scanning, point scanning, single slit and multiple slits subtraction microscopes. (d) Same on a log-log scale. The multi-stripe subtraction system is here equivalent to the structured illumination microscope.

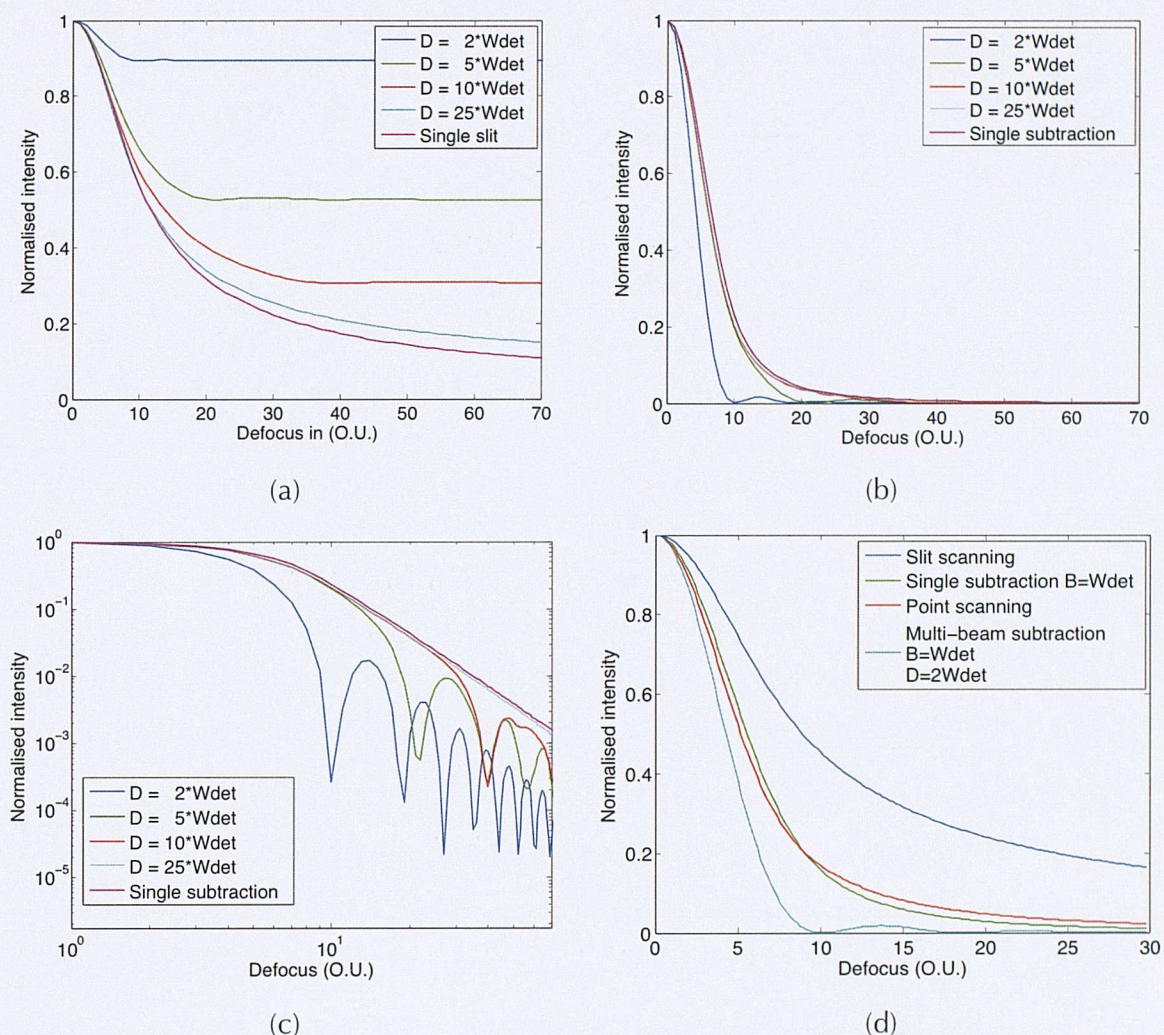


Fig. 6.7. Simulations of a multi-line subtraction microscope. Illumination and detection slit widths are assumed to be $W_{ill}=17\mu\text{m}$ and $W_{det}=34\mu\text{m}$, respectively and $\beta=W_{det}$, which corresponds to the stripe LED centre-to-centre spacing.. (a) Multi-slit signal before background subtraction. (b) Multi-slit signal after subtraction. (c) Same data on a log-log scale. (d) Slit, point, single slit subtraction and multiple slit subtraction sectioning.

3. Design of a double-sided spinning disk system for high-speed confocal imaging with improved optical sectioning

Because the multi-line subtraction system is fairly similar to an already existing double-sided spinning disk microscope whose rotating pattern is composed of densely packed lines [8], it is of interest to compare the sectioning performances of both systems and their corresponding advantages. This leads to the design of an improved double-sided spinning disk that should enable high frame rate confocal imaging with superior optical sectioning.

3.1. Optical sectioning of double-sided spinning disks

In the high fill-factor illumination microscope, the double-sided disk is located in critical illumination such that the line pattern is imaged onto the sample (Fig. 6.8 (c)). The in-focus reflected/fluorescence light coming back from the object is routed back through the disk and forms an image I_1 on a first camera (CCD1 in Fig. 6.8 (c)). In the meantime the out-of-focus contribution I_2 is reflected on a complementary part at the back of the same disk towards another camera (or a different location on the same camera). Conventional and sectioned images can be calculated using the following equations:

$$\begin{aligned} I_{\text{sec}}(u) &= I_1(u) - \alpha I_2(u) \\ I_{\text{conv}}(u) &= I_1(u) + \alpha I_2(u) \end{aligned} \quad (6.17)$$

where α is a subtraction scaling factor, whose impact on the image quality is discussed later. Let's consider first the spinning disk depicted in (Fig. 6.8 (b)) operating in fluorescence. Because the same set of slits is used for illumination and detection, the response to defocus in I_1 can be written

$$\begin{aligned} I_1(u) &= p^2 \int_{-\infty}^{+\infty} T^2(s, u) \text{sinc}^2\left(\frac{ps}{2\pi}\right) \left[\sum_{k=-N}^N e^{ik\delta s} \right] ds \\ &= v^2 p^2 \sum_{0 \leq k \leq \frac{2}{v}} T^2(kv, u) \text{sinc}^2\left(\frac{kp v}{2\pi}\right) \end{aligned} \quad (6.18)$$

where δ and v are the normalised period and frequency of the line pattern, as defined in the previous section.

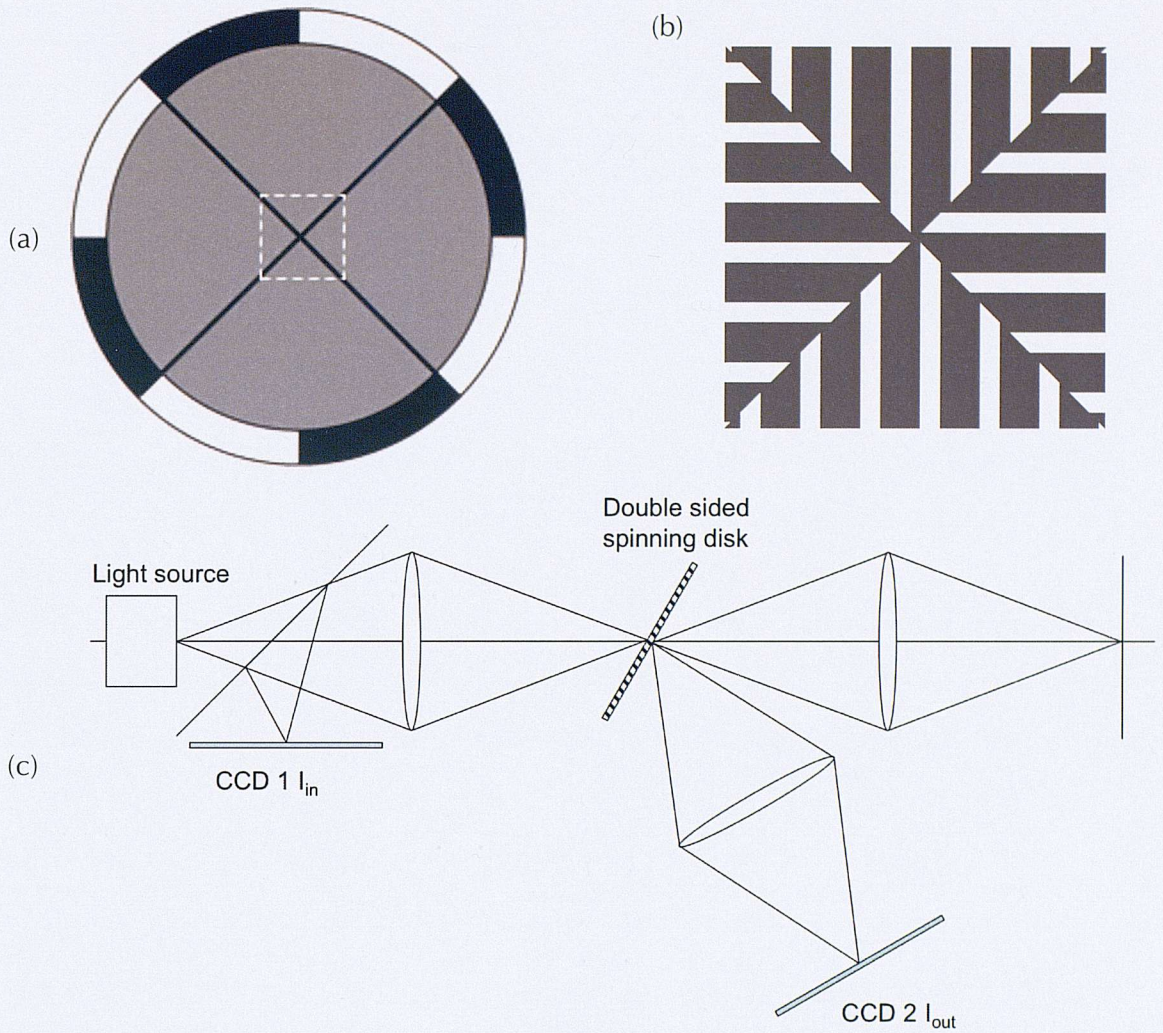


Fig. 6.8. Double-sided spinning disk microscope. (a) Disk. (b) Zoom of the highlighted area in (a) showing a the disk structure. The grey area reflects the out-of-focus light towards CCD2. (c) Confocal microscope setup.

On the other hand, I_2 is composed of the light originating from the illumination slits of width p and captured by the slits q translated by the normalised distance β , giving an out-focus signal:

$$I_2(u, \beta) = 2v^2 pq \sum_{0 \leq k \leq \frac{2}{v}} T^2(kv, u) \operatorname{sinc}\left(\frac{kp v}{2\pi}\right) \operatorname{sinc}\left(\frac{kq v}{2\pi}\right) \cos(k\beta v) \quad (6.19)$$

Introducing both equations (6.18) and (6.19) into Equation (6.17) gives

$$I_{\text{sec}}(u, \beta) = v^2 \sum_{0 \leq k \leq \frac{2}{v}} T^2(kv, u) \text{sinc}^2\left(\frac{kpv}{2\pi}\right) p^2 \left[1 - 2\alpha \frac{q}{p} \frac{\text{sinc}\left(\frac{kqv}{2\pi}\right)}{\text{sinc}\left(\frac{kpv}{2\pi}\right)} \cos(k\beta v) \right] \quad (6.20)$$

Because only the zero order ($k=0$) is transmitted when $u \rightarrow \pm\infty$, the intensity at large defocus can be written

$$I_{\text{sec}}(u \rightarrow \infty) \propto T^2(0, u) \left[1 - 2\alpha \frac{q}{p} \right] \quad (6.21)$$

which is constant with defocus. This suggests the value $\alpha = p/2q$ for best sectioning. The factor α represents the ratio of the energies in the transmitted and reflected images. It can also be noted that the intensity remains constant throughout focus when adding both images, which corresponds to the widefield case.

Considering a spinning disk where the whole space between two slits is reflecting the out-of-focus light, it is clear from the Fig. 6.8 (b) that the grid period δ and detection phase β can be expressed using the illumination and detection widths p and q .

$$\begin{aligned} \delta &= p + 2q \\ \beta &= \frac{p + q}{2} \end{aligned} \quad (6.22)$$

The response of this microscope to a fluorescent planar object can thus be written

$$I_{\text{sec}}(u, \beta) = v^2 \sum_{0 \leq k \leq \frac{2}{v}} T^2\left(k \frac{2\pi}{p + 2q}, u\right) \text{sinc}^2\left(\frac{kp}{p + 2q}\right) p^2 \left[1 - \frac{\text{sinc}\left(\frac{kq}{p + 2q}\right)}{\text{sinc}\left(\frac{kp}{p + 2q}\right)} \cos\left(k\pi \frac{p + q}{p + 2q}\right) \right] \quad (6.23)$$

Results from this simulation are analysed later and compared to a new disk design described in the following section.

3.2. Effect of the blocking area

Of course this microscope geometry is very similar to the multi-stripe LED system that was investigated in section 2. However, the basic disk structure has detector slits that completely fill the space between the illumination slits. This results in a poorer haze rejection. This section analyses the haze rejection capability of the first disk and

proposes a modification to the disk patterning to provide something similar to the actual multi-stripe geometry of section 2, where the detection slits are similar in size to the illumination slits. To achieve this, a light blocking area is introduced between the stripes on the disk as shown in Fig. 6.9 in order to narrow the out-of-focus detection slits. The impact of the blocking area on the sectioning strength is now investigated.

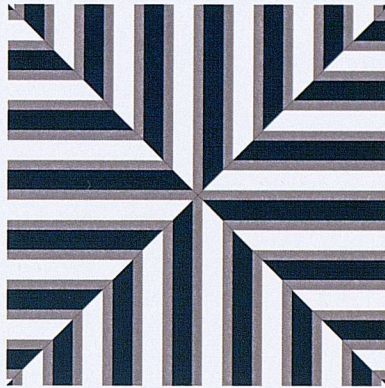
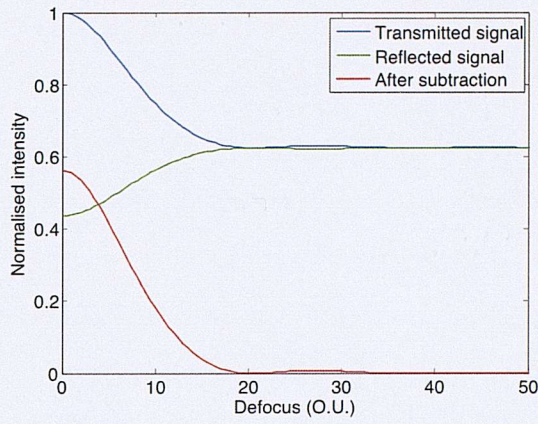
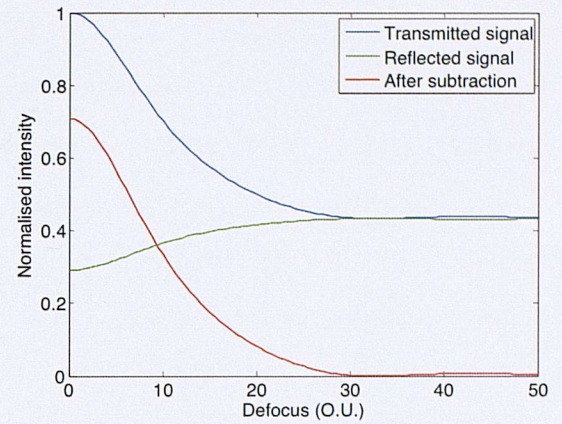
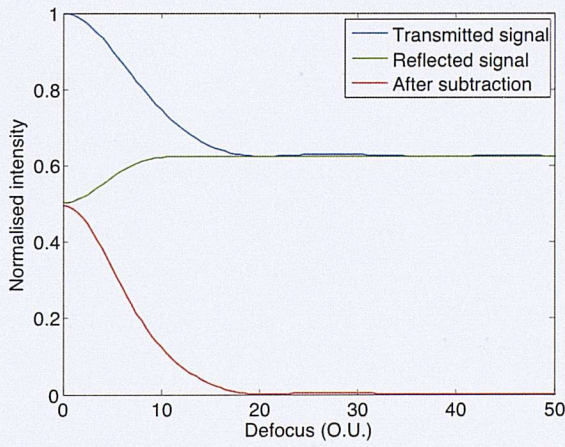
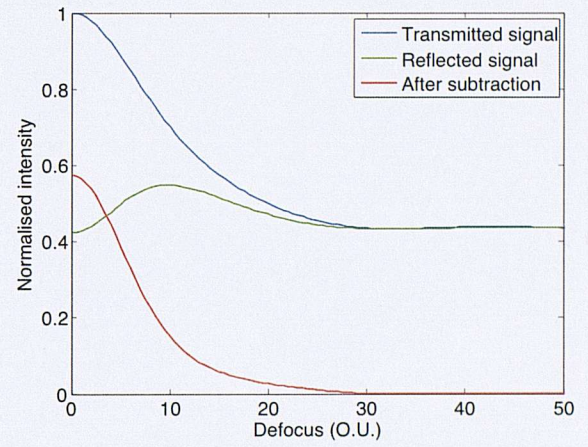
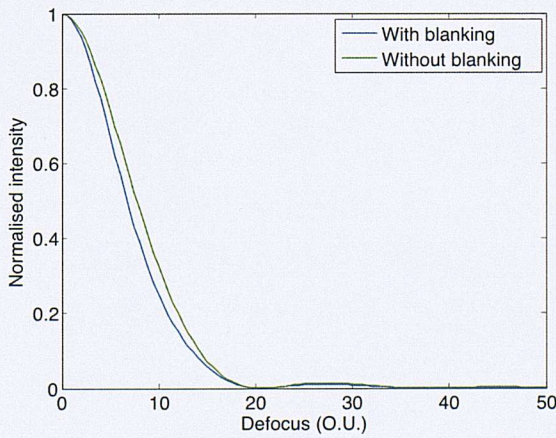


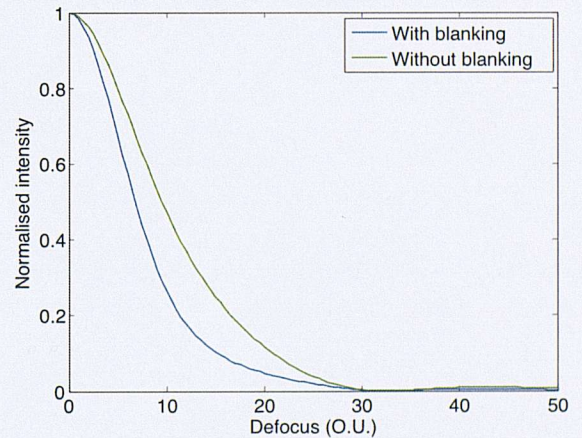
Fig. 6.9. 30% fill-factor line patterns used to acquire high-speed confocal images. In the disk patterns, the white areas transmit the light coming back from the sample towards the CCD1, the grey areas reflect the out-of-focus light towards CCD2 and the black areas absorbs the light.

Simulations were made with $q = (\delta - p)/2$ in case of a conventional disk and $q = p/2$ (to ensure that $\alpha=1$) in case of a partially blocking disk. Results shown in Fig. 6.10 suggest that narrowing the reflecting slits improves the sectioning capability of the microscope at the expense of a slightly smaller signal after background subtraction. However, when the reflecting slits become too narrow ($2q < p$), the scaling factor α becomes bigger than unity and the noise from the out-of-focus region is amplified before image subtraction.

The effect of the blocking area becomes prominent when decreasing the illumination fill-factor, as shown in Fig. 6.10 (f), because the information about where the out-of-focus light originates is lost as q increases.

(a) Conventional disk: $\delta=3p=3q$ (d) Conventional disk: $\delta=5p=5/2 q$ (b) Blocking disk: $\delta=3p=6q$ (e) Blocking disk: $\delta=5p=10q$ 

(c)



(f)

Fig. 6.10. Impact of the blocking area on the spinning disk microscope sectioning strength ($p=10$ O.U.). (a)-(b) Transmitted light, reflected light and sectioned signal for a disk with $\delta=3p$ (a) with no blanking ($q=(\delta-p)/2$) and (b) with blanking ($q=p/2$). (c) Normalised sectioned signals for $\delta=3p$.-(d)-(e) Same as (a)-(b) but for $\delta=5p$. (f) Normalised sectioned signal for $\delta=5p$.

3.2.1 Effect of the size of the reflecting slits

As α increases (q is decreased while p remains the same), the signal in the sectioned image decreases (Fig. 6.11 (a)) and the sectioning improves and finally converges towards the diffraction limited case when $q=\pi$. This is illustrated in Fig. 6.11 (b) for $p=10$ O.U. and $\delta=5p$. There is no point of narrowing the reflecting slit below $q=\pi$ O.U. as this represents the smallest object that can be resolved with the objective lens. Sectioning would not improve further, only fewer photons would be collected, leading to a lower signal to noise ratio in the sectioned images.

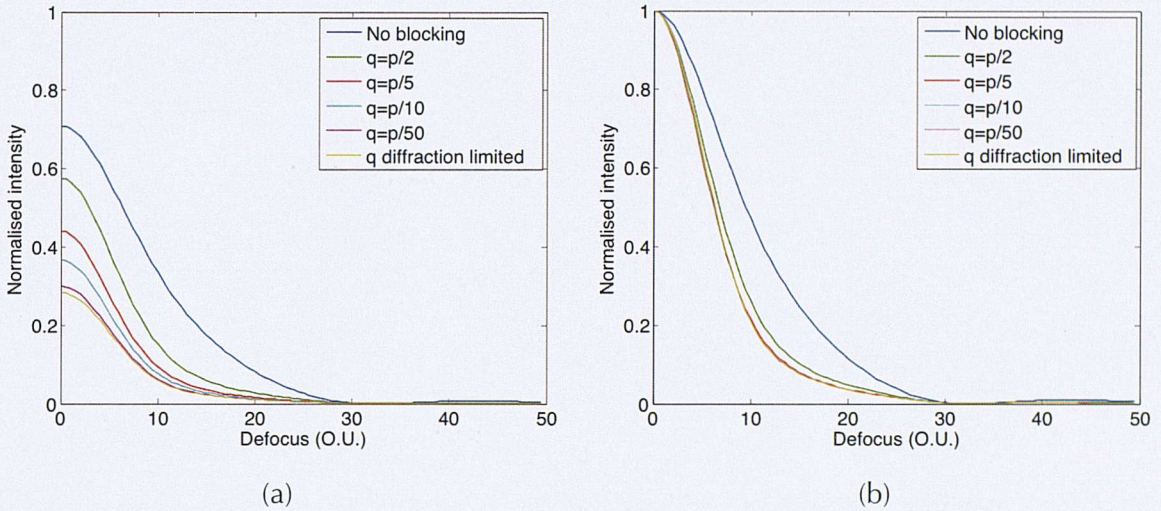


Fig. 6.11. Effect of varying the size q of the reflective slits. (a) Sectioned signal I_{sec} normalised by the maximum intensity in the transmitted image I_t . (b) Normalised responses to defocus. Here $p=\delta/5=10$ O.U.

3.2.2 Effect of the finite well-capacity of the detector

Assuming both raw images I_1 and I_2 are recorded at different locations on the same CCD detector, the dynamic range of the sectioned image is limited by the brightest raw signal. For $\alpha=1$ the image I_1 containing the in-focus contribution will always overcome the signal I_2 . However as α decreases, the raw out-of-focus signal I_2 will have to be scaled down by α before subtraction and might then overcome the in-focus signal. The signal I_2 would then limit the dynamic range in the sectioned image. The reduction of dynamic range in the sectioned imaged compared with the dynamic range of the camera can be calculated as

$$D(I_{\text{sec}}) = \frac{\text{Max}(I_{\text{sec}})}{\text{Max}(I_1, I_2)} \quad (6.24)$$

Fig. 6.12 shows this reduction factor for a spinning disk microscope with and without blocking area on the disk and for different grid periods. When δ is relatively small, the higher sectioned signal of the non-blocking disk results in a better dynamic range compared to the blocking disk. However, this degrades very soon after because the amount of out-of-focus light detected by the non-blocking disk increases proportionately with q .

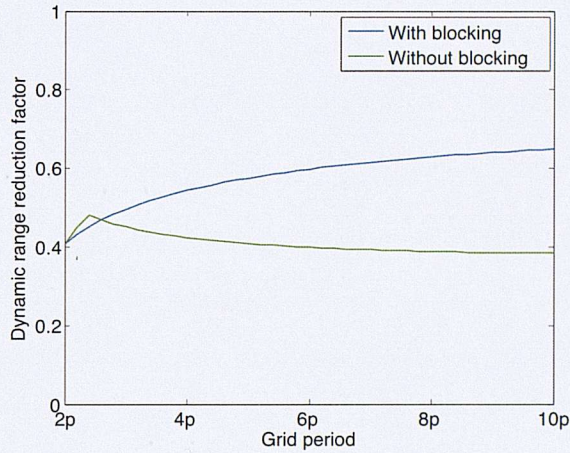


Fig. 6.12. Dynamic range reduction in the sectioned image compared with the camera dynamic range for a spinning disk with and without blocking areas ($p=10$ O.U.)

3.3. Noise in double sided disk microscopes

3.3.1 Noise floor

From Equation (6.17) and assuming a normally distributed photon noise, the uncertainty in the sectioned image is given by

$$\Delta I_{\text{sec}} = \sqrt{I_1 + \alpha^2 I_2} \quad (6.25)$$

As seen in Fig. 6.13 for a spinning disk with blocking area with $q = p/2 = 5$ O.U., the value of the noise floor decreases together with an signal increase as the line pattern period is increased. However a lower fill-factor goes at the expense of a lower light throughput. Thus, for a given camera acquisition time, the illumination intensity would need to increase proportionally.

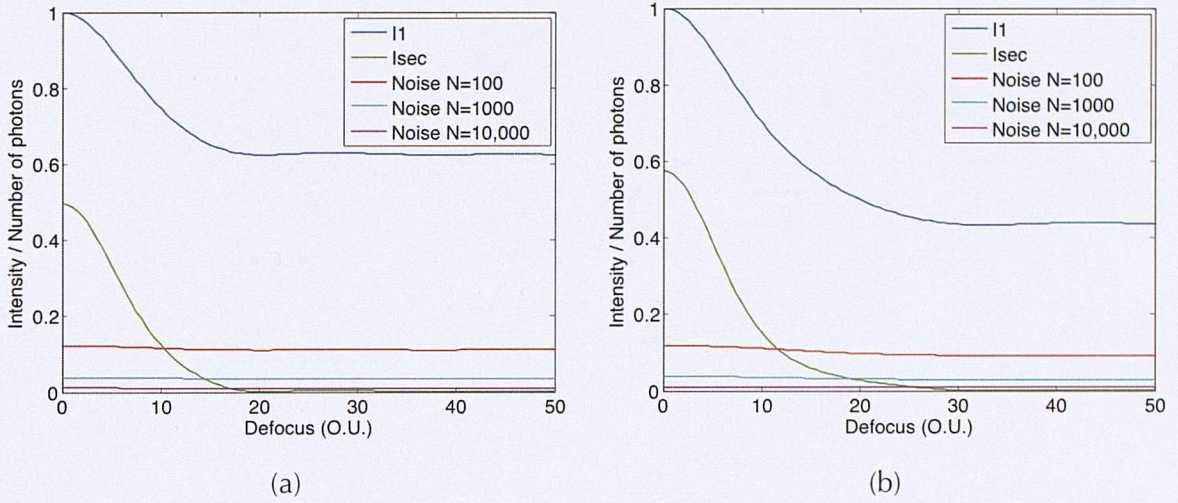


Fig. 6.13. I_1 , I_{sec} and noise floors for a spinning disk for several numbers of detected photons. (a) $\delta=3p$ and (b) $\delta=5p$.

3.3.2 Impact of a bright out-of-focus object on the signal to noise ratio

Let's now consider a fluorescent object composed of two planes, one in-focus and the other far out-of-focus. Until now, the response with defocus was characterised qualitatively. However, in order to derive noise expressions for the microscope, quantitative measurements have to be taken into account. Considering a fluorescent object, the illumination is usually three orders of magnitude brighter than the fluorescence emission due to the inherently low fluorescence yield. Therefore the noise arising from the illumination can be neglected compared with the noise from the detection. The transmitted image then can be described as

$$\begin{aligned} I_T &= I_1(u=0) + I_1(u \rightarrow +\infty) \\ &= I_1(0) + v p I_0 \end{aligned} \quad (6.26)$$

where $I_1(0)$ is the contribution of the in-focus object to the transmitted image and I_0 is the intensity of the out-of-focus object. Similarly, the intensity in the reflected image can be written

$$\begin{aligned} I_R &= I_2(u=0) + I_2(u \rightarrow +\infty) \\ &= I_2(0) + 2v q I_0 \end{aligned} \quad (6.27)$$

From Equation (6.17), the uncertainty in the sectioned image can be expressed in terms of the normally distributed measurements in the transmitted and reflected images:

$$\begin{aligned}\Delta I_{\text{sec}}^2 &= \Delta I_T^2 + \alpha^2 \Delta I_R^2 \\ &= I_1(0) + vpI_0 + \alpha^2 I_2(0) + 2\alpha^2 vpqI_0\end{aligned}\quad (6.28)$$

giving a signal to noise ratio of

$$SNR = \frac{I_1(0) - \alpha I_2(0)}{\sqrt{I_1(0) + vpI_0 + \alpha^2 I_2(0) + 2\alpha^2 vpqI_0}} \quad (6.29)$$

Using a disk with blocking areas ($p=2q$ and $\alpha=1$) and assuming that the out-focus image is a factor γ times the in-focus contribution $I_0 = \gamma(I_1(0) + I_2(0))$, the SNR can be written

$$\begin{aligned}SNR &= \frac{I_1(0) - I_2(0)}{\sqrt{(I_1(0) + I_2(0))(1 + 2\gamma vp)}} \\ &= \frac{SNR_0}{\sqrt{1 + 2\gamma vp}}\end{aligned}\quad (6.30)$$

The product vp represents the fill-factor of the disk. SNR_0 is the SNR in the absence of an out-of-focus object and only depends on the diffraction properties of the disk. A graph of how much the SNR is reduced relative to SNR_0 is shown in Fig. 6.14 as a function of both the fill factor and γ . It is clear that there is no strong argument for reducing the fill factor as the signal to noise ratio does not increase very quickly. For instance the SNR increases by a factor 2.15 for $vp=0.1$ when $\gamma=50$ over $vp=0.5$.

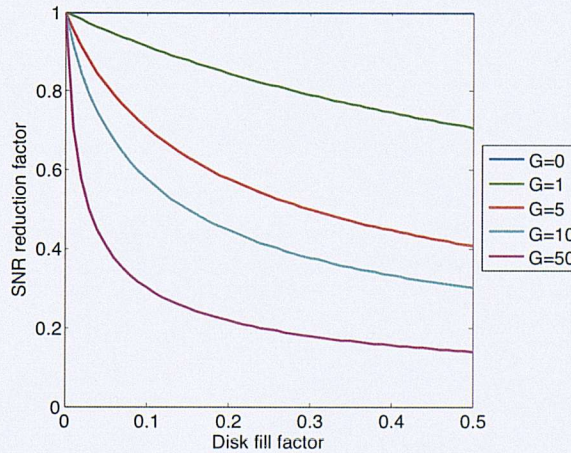


Fig. 6.14. Graph of the reduction in SNR as a function of both γ (G in the figure) and vp for the case where the number of photons to be detected from the in-focus image is the limiting factor.

The $\sqrt{1 + 2\gamma vp}$ reduction factor in SNR over SNR_0 does not take into account the diffraction properties of the disk but only the effects of an out-of-focus object

together with the geometrical aspect of the disk. In order to derive the real SNR, one has to calculate SNR_0 , which varies significantly with the size of the illumination and detection slits.

4. So, which microscope to choose?

For most of the microscope configurations described earlier, no optimal setup exists, but only a reasonable trade-off can be achieved. The method employed to achieve optical sectioning, either structured illumination or spinning disk microscopy, the illumination fill-factor and the sizes of the illumination and detection slits should be chosen according to the light source which is available, the brightness, thickness and scattering properties of the sample and the performance of the CCD camera. However, the properties of the structured illumination systems for different illumination fill-factors can be summarised as followed:

Illumination fill factor	Advantages	Weaknesses
High	- Higher photon efficiency (Faster acquisition or dimmer light source) - Superior optical sectioning	- Lower SNR - Higher sensitivity to sample scattering - Higher sensitivity to optical aberrations
Small	- Higher SNR - Lower sensitivity to sample scattering (True confocal effect)	- Lower photon efficiency - Marginally degraded optical sectioning

5. Summary

The theory of optical sectioning for a slit scanning confocal microscope with background subtraction was presented. The subtraction technique is shown to dramatically improve axial resolution over the conventional line scanning system and yields a marginal resolution improvement over the point-scanning system. The same subtraction method to reject out-of-focus light is applied to multi-slit images. This method was described to fill the gap between line-scanning microscopy and

structured illumination microscopy. Performances of a multi-line subtraction system in presence of photon noise were characterized. Based on these observations, a new double-sided spinning disk was designed in which a blocking area was included on the reflecting side of the disk between adjacent detection slits. This design allows for superior optical sectioning, especially when large mark/space ratio disk patterns are to be used due to the large signal originating from the out-of-focus part of the sample.

Chapter 7 Application of LED arrays to neurophysiology

Since the original discoveries by Hodgkin and Huxley in 1952 [123] neurobiologists have gained considerable insight into the functioning of neurons, thanks to evolving techniques to excite neuron responses while recording neural activity. Stimulating electrodes have been a valuable tool as they allow for electrical control of neurons on a millisecond time scale. However, direct electrical excitation of neuron cells is difficult and poses two main challenges. First, extracellular electrodes have a poor spatial discrimination, making individual cell and defined multipoint excitation difficult to apply. Second, electrical stimulation and recording relies on mechanical stability and is often challenging for in-vivo animal experiments. While electro-stimulation techniques have been continuously improved, several light-based stimulation techniques have also been developed.

The first category is via the photolysis of caged neurotransmitters, which was also the first modern photo-stimulation technique [124]. Neurotransmitters rendered inactive with covalently bonded blocking ligands are released into the solution around the neurons. The blocking moiety bonds with the neurotransmitters are then broken with the use of UV light, which can then stimulate the neurons [125]. The second category involves the use of artificial membrane photoswitches. This is a relatively new technique that uses synthetic photoisomerizable nano-compounds to block or open ion channels or receptor proteins [126]. The final and perhaps the most promising category is the expression of natural phototransduction membrane proteins [127], and this technology is the main focus of investigation in this chapter.

Light based stimulation techniques offer many advantages over traditional electro-stimulation technologies. The light beam can be easily focused to very high spatial resolution using conventional optics, limited only by diffraction of the light, and individual neuron types in a heterogeneous culture can be selectively stimulated by using genetic targeting with appropriate photosensitization agents. The location of the stimulating beam can be easily altered during the experiment and action potentials

can also be inhibited by the use of photo-inhibiting agents that are sensitive at different wavelengths to the photo-stimulating agents [128, 129].

At present, however, stimulating neurons with light is limited to a single stimulating signal. This is because the light intensity and the spatial resolution that are required for the stimulation process can only be matched today by high power illumination sources such as Xenon/Mercury lamps [130], lasers [131] or high power light emitting diodes (LED) [132] combined with a microscope or optical fibre setup.

This chapter introduces a multi-site, multi-signal light stimulation platform, based on the micro-LED array technology. First the capability of UV micro-arrays for photo activating micro patterns on a caged fluorophore sample is demonstrated. We then move on to investigate an ion-channel stimulation system based on a genetically expressed, blue light sensitive membrane protein, Channelrhodopsin-2 (ChR2), which was recently discovered in photosensitive algae. Sub-cellular stimulation of photosensitised neurons is then carried out and an integrated system for photo-stimulation and electrophysiological recording of photosensitised cell activity is presented. The resulting system allows the simultaneous optical stimulation of 2 dimension patterns on a biological neuron network with electronic recording via a microelectrode array plate or fluorescent imaging of calcium across the membrane. In this manner, biological neural networks can be probed at the network level. Finally, a longer term project to use LED arrays in LED-based retina prosthetics is presented. The prosthetic is non-invasive and should be able to provide augmented vision after photoreceptor apoptosis.

Much of the work presented in this chapter was carried out in collaboration with the Institute of Biomedical Engineering (IBE) at Imperial and the Department of Neurophysiology at Kings College London. In particular the assistance of Nir Grossmann from the IBE is acknowledged in the experiments involving neurons that are presented in this chapter.

1. Flash photolysis of caged-compounds

1.1. Photo-activation techniques

The principle of photo-caging is that a molecule of interest can be rendered inert by the chemical addition of a photo-removable protecting group. Flash photolysis is the

activation of this caged-molecule in a sample using a beam of light. Under intense irradiation at a particular activation wavelength, the caged or trapped molecule is liberated to induce a local perturbation of a biological process. The uncaging light beam is a powerful tool for controlled stimulation of biological targets in space and time. Many examples of caged molecules can be found in the literature [133], especially in neuroscience where important release compounds, such as calcium [134] and neurotransmitters [135], are used on a regular basis to study secretory processes [136] and trigger neural activity [135, 137].

1.1.1 Uncaging techniques and light sources

The vast majority of caged molecules are released under UV light illumination. Pulsed xenon or shuttered mercury lamps are therefore commonly used to directly achieve uncaging homogeneously across the specimen. In a confocal microscope, photolysis can be achieved using a near-UV laser [131]. However, conventional microscope lenses do not perform well in the UV region and UV lasers are often expensive to purchase and to maintain. Alternatively ultrafast pulsed near-IR illumination can be used to break these UV bonds by two-photon absorption. The use of two-photon IR excitation also offers some potential intrinsic advantages in terms of depth of uncaging in tissues and axial confinement [138]. The two-photon effect ensures that molecules are only released in the focal plane, whereas the energy conservation under direct UV uncaging implies that the same number of molecules is released at every depth in the sample. This results in a non-localized photolysis and in an out-of-focus blur when imaging the uncaged molecules with a widefield microscope.

1.2. Fluorescein uncaging using UV LED arrays

Photo-uncaging systems that release a fluorescence probe have also found use in a number of areas. Fluorescein is a widely used fluorophore due to its large cross-section and strong fluorescent signal. In its caged form it has been extensively used in cell imaging and microfluidics measurements [139, 140]. The fluorescein is attached to the compound 5-carboxymethoxy-2-nitrobenzyl that hinders its fluorescence, but can be released by UV flash photolysis. Fluorescein uncaging was recently demonstrated using a single 100mW diode emitting at 365nm coupled into an optical fibre [141]. The fibre was directly positioned in close proximity to the sample of

interest, resulting in a 100 μ m uncaging spot. This system was also successfully employed to stimulate neurons by photolysis of caged-glutamate [141], although the low spatial resolution and mechanical considerations made it impractical for routine use.

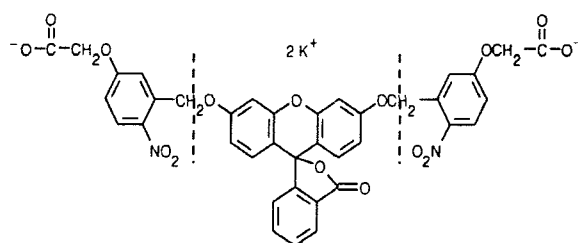


Fig. 7.1. Molecule of caged-fluorescein. The left and right compounds, whose bonds (blue lines) are cleaved under UV illumination, hinder fluorescence.

In order to prove the capability of the new micro-LED array to realize a structured photo-uncaging pattern, the uncaging of CMNB-caged fluorescein (Molecular Probes) was triggered with a stripe UV micro-LED array that emits at 370 nm.

1.2.1 *Experimental conditions*

The 370nm top-emitting micro-stripe diode was placed in critical illumination in an Olympus BX41 upright microscope so that the pattern displayed on the LED array is imaged onto the sample using a 20X 0.5NA objective. A 470nm Luxeon® V LED is placed in Kolher illumination to excite the fluorescence from uncaged molecules and coupled into the microscope using a 50/50 beam-splitter (Fig. 7.2). The blue LED is mounted on an appropriate heat sink and driven at 1A when exciting fluorescence. Uncaging UV light was projected onto the sample using a UV Olympus dichroic and fluorescence from the sample was observed using a separate standard blue fluorescence filter cube. In this configuration the units had to be switched between the UV uncaging phase and the blue observation phase. The arrangement of light sources did not require an extra dichroic mirror to direct both the UV illumination and the blue light into the microscope, although it did result in a loss of 50% of each in terms of power.

The CMNB-caged fluorescein was dissolved in 100% glycerol (100mM) in order to reduce the diffusion of the molecules. The solution was then spin-coated onto a plasma treated microscope cover slips to produce uniform thin films. During the

uncaging experiment UV light was shone onto the sample for a period controlled by the LED driver. The filter cube was then switched from UV to blue, the blue LED was turned on and the fluorescence light was imaged onto a CCD camera (Orca ER). The diode elements were driven at 5mA under 10V maximum forward voltage, resulting in an average power of 34nW per emitter on the sample.

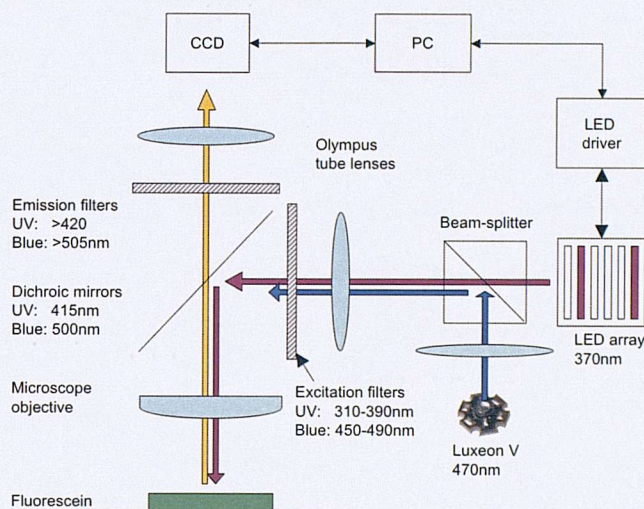


Fig. 7.2. Microscope configuration for patterned photo-uncaging.

1.3. Results

1.3.1 *Caged fluorescein*

The caging ligands have peak sensitivity at 330 nm (3.8 eV). Thus, the 370 nm wavelength (3.4 eV) emitted by the UV GaN arrays was not optimal. To make sure the UV diode could uncage fluorescein, photolysis was tested in a cuvette using a spectro-fluorometer (Shimadzu RF-5301PC) to illuminate the sample at $\lambda=370\text{nm}$ and record the fluorescence spectrum of the uncaged compound under 470nm blue excitation, before and after UV irradiation. Additionally the 100% Glycerol solvent was checked not to exhibit any fluorescence under blue light illumination.

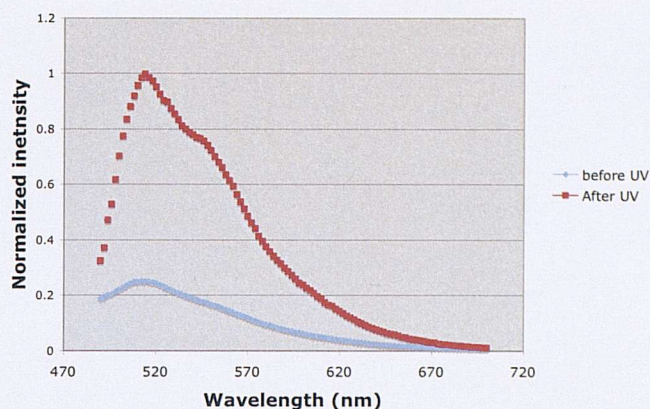


Fig. 7.3. Fluorescein emission spectra before (blue) and after UV illumination (red).

Fig. 7.3 shows a resulting 4-fold increase in the fluorescence signal after low-level UV illumination, with an emission peak in both cases at 513 nm.

1.3.2 Uncaging experiment

A random pattern of stripes was projected onto the caged fluorescein sample as shown in Fig. 7.4 (a). The resulting fluorescence patterns acquired after 34, 85, 170 nJ per stripe (corresponding to 1, 2.5 and 5 seconds UV exposure, respectively) are shown in the remaining images (c) to (e). Approximately 100 nJ was required to photolyse a significant visible fluorescent pattern with the current setup and the increase in fluorescence signal was approximately linear with excitation dose. In this set-up, the width of a single stripe in the image was approximately $1\mu\text{m}$, although the corresponding un-caged width in the image was wider ($\sim 5\mu\text{m}$) due to diffusion of uncaged molecules and uncaging occurring outside the focal plane. Based on a $1\mu\text{m}$ projected stripe width and a $180\mu\text{m}$ projected strip length, this 100nJ total exposure corresponds to a dose per unit area of $560\text{pJ}/\mu\text{m}^2$.

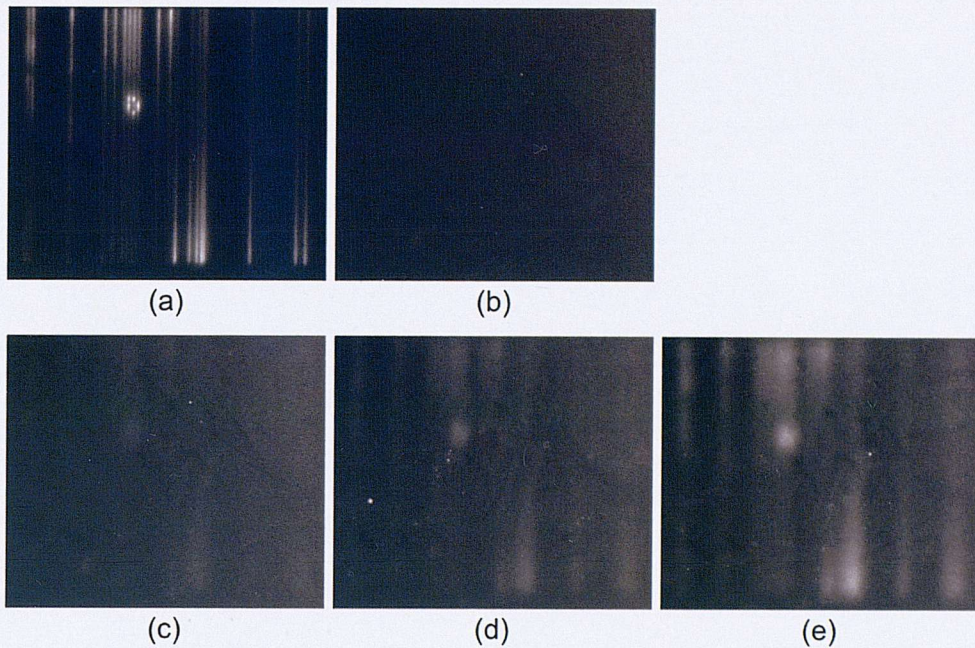


Fig. 7.4. Uncaging of caged fluorescein using a UV stripe array. (a) Pattern displayed on the diode, (b) Before UV, (c) after 1s UV, (d) after 2.5s UV and (e) after 5s UV.

The UV micro-stripe LED array chip used in the experiment was a prototype device and therefore still had some significant non-uniformity in emission. Stripes appeared brighter near their connection end and gradually dimmer away from it, as in the first generation series of stripe devices. Several LED stripes did not emit at all mainly due to broken contacts at the surface of the semiconductor. However, stripes that did work showed very good consistency in their average optical power when driven at constant current. The average stripe power at the diode was 34 nW and the standard deviation was 2.25 nW (measured in the sample plane using a Newport 818-UV power meter). The uniformity is better in the pixellated LEDs chips due to a metal electrode reducing the resistance along each mesa, however only stripe format devices were available at UV wavelengths at the time of the experiment.

1.4. Discussion

The experiment described above demonstrated the feasibility of using micro-LEDs in an array format to activate CMNB-caged fluorescein. This work highlights how micro-LED arrays could be used to photo-release of neurotransmitters to trigger neuronal activity. For neurotransmitter uncaging, the blocking moiety is similar that of caged-fluorescein (typically based on the nitrobenzyl group). Photolysis of signalling

molecules is a common approach to excite neurons. Membrane potential or cellular signalling can be modulated when light releases a blocking compound covalently attached to an active molecule, such as glutamate, Gamma-aminobutyric acid (GABA) or secondary messengers such as Ca^{2+} . UV light can be used to uncage glutamate in the vicinity of targeted neurons for activating endogenous receptors, which can result in a neuron action potential. In similar work using a single large area light source, photo activation of caged-glutamate required less optical power than caged-fluorescein due to the high sensitivity of the neurons to low neurotransmitter concentrations. Recently researchers used glutamate uncaging to probe the cellular connectivity of a neural network by exciting neurons at several hundred sites [142]. Neurons could be stimulated with a temporal resolution of several tens of milliseconds and a spatial resolution of fifty microns.

Photo-uncaging is only one method by which photoactivation of neurons can be achieved. The rest of this chapter now discusses a different mode of photoactivation via the genetically expressed, blue light sensitive membrane protein, Channelrhodopsin-2 (ChR2).

2. Sub-cellular photo-stimulation of neuronal activity

The study of neural activity can be undertaken at different scales, depending on the issue to be investigated. For certain problems, it is important to record neuron signals from single neurons or even a single ion channel in a neuron, while stimulating or inhibiting either a precise part of the cell or even several parts simultaneously. For recording, patch clamping has been an effective tool to investigate the biochemical factors involved in neural signalling [143]. In this chapter, micro-structured LEDs are used for the first time here as a light source for neuron studies by combining both sub-cellular photo-stimulation and patch clamp recording of individual cells.

2.1. Photosensitized neurons

A very promising technique for photo-stimulation of neuronal activity is via the expression of natural phototransduction membrane proteins. They come in two forms: light activated cascades leading to ion channels opening and individual light gated channels.

2.1.1 Melanopsin

It was recently discovered that our ability to tune our body clocks to days and night relies on a protein, the Melanopsin, present in small subset (~1%) in non-image forming ganglion cells in the eye [144, 145]. It was previously believed that the cones and rods were the only photoreceptors present in the eye, while ganglion cells were only important at the initial stage of visual processing and in conveying the information off the retina to the optic nerve system. However it appeared that the loss of these known photoreceptors has little effect on animals' ability to tune their circadian system, whereas, a complete loss of the eye abolishes this ability [146, 147]. Melanopsin is a G-protein type photopigment, which has a maximum sensitivity in the blue region of the spectrum, at a wavelength of 479nm [148]. It was subsequently shown that the addition of human melanopsin renders mammalian cells photo responsive [149]. The G-protein cascade process makes neurons expressing melanopsin very sensitive to light, which can be understood on the evolutionary point of view as the need for the eye to detect dawn and dusk low light levels. However, from an engineering perspective the cascading effect results in a high sensitivity of the photosensitive cells to ambient blue light, which might interfere with the usual visual system.

2.1.2 Channelrhodopsin-2 (ChR2)

Channelrhodopsin-2 (ChR2) is a direct light gated cation channel found in the *Chlamydomonas reinhardtii* algae [150], which too is activated with blue light. When expressed in mammalian cells, brief photo-stimulation is capable of generating inward cation currents and if enough ion channels open at once, the depolarization of the membrane induces an action potential. These electrical spikes have been shown to be indistinguishable from the natural spikes [130] that occur in the normal visual system. By using light pulses as short as 10ms, reproducible trains of spikes can be triggered. Because ChR2 has no amplifying cascade process, cells are excited with blue light which must be of the order of a thousand-fold brighter than ambient light. The advantage is the resulting very low toxicity of ChR2, as it remains closed unless it is excited by bright blue light. This is also a clear advantage from the engineering point of view as there is no need to avoid background light.

2.1.3 Kinetics

ChR2 is rapidly activated by blue light and has a fast deactivation time constant [130, 151], which makes it particularly attractive for generating fast neuron spikes. It was generally believed that ChR2 could be described as a 3 state system (Fig. 7.5). In the Closed state the protein is at rest and the absorption of a photon induces a conformation change, which opens the channel. In the Open state cations can flow through the membrane and an inwards current can be measured. In the Deactivated state the channel is closed but the protein cannot be further excited until it relaxes back to the Closed state. The time constants for the three states have been measured to be 0.2ms for opening, 20ms for deactivation and between 2 to 6 seconds for recovery (τ_c in Fig. 7.5). A more rigorous kinetics model, in which the protein has two deactivation dark-adapted and light-adapted pathways with two different lifetimes, is currently under investigation [151].

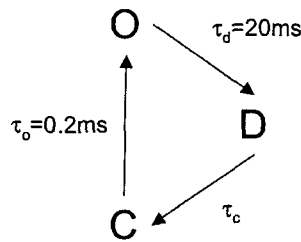


Fig. 7.5 Simple three-state model of ChR2 photo-cycle.

2.1.4 Light requirements

Light sources must be carefully matched to the photo-stimulation technique used. ChR2 is optimally activated with blue light with an excitation maximum between 460 and 470nm, which makes GaN LEDs ideal candidates for neuro-stimulation.

Boyden *et al.* [130] and Ishizuka *et al.* [132] reported that a power density of 5-12 nW/ μm^2 was required to stimulate hippocampal culture cells expressing ChR2 [130, 132]. While, Bi *et al.* [152] reported later that a power density of only 50 pW/ μm^2 was sufficient to stimulate ChR2 expressing mice retinal neuron cells [152] - a difference of three orders magnitude. This may be attributable to different levels of ChR2 expression (not specified in the texts) or due to the different type of cells that were used. In comparison to ChR2, the activation of melanopsin requires a

much lower irradiance - approximately $0.5\text{-}5\text{ pW}/\mu\text{m}^2$. This is because the G-protein cascade amplifies the ion channel opening process.

2.1.5 *Temporal and spatial control*

Precise temporal control of electrical activity using ChR2 also depends on the ability to control the light exposure on the millisecond timescale. This can be accomplished in several ways, including ultra-fast mechanical or optical shutters or by high-speed flashing of an LED. The excitation powers required to activate ChR2 make it possible to use these convenient light sources for neural excitation.

2.2. System for dynamic sub-cellular photo-stimulation

2.2.1 *Microscope setup*

Micro-LED arrays were employed as illumination sources for sub-cellular stimulation of neurons transfected with ChR2. An extra LED module was constructed and mounted on top of an inverted microscope equipped with a patch-clamp recording system. The patterned LED array is controlled by the computer and projected onto the sample using a 4f relay system (or a water dipping objective lens when demagnification is required). The genetically expressed ChR2 is tagged with a fluorescent protein (such as YFP or m-Cherry) and a collimated Xenon lamp is attached at the rear of the microscope to excite fluorescence emission. The fluorescence image is captured using a CCD camera, which allows for monitoring of the cell transfection and the spatial distribution of ChR2 gene expression.

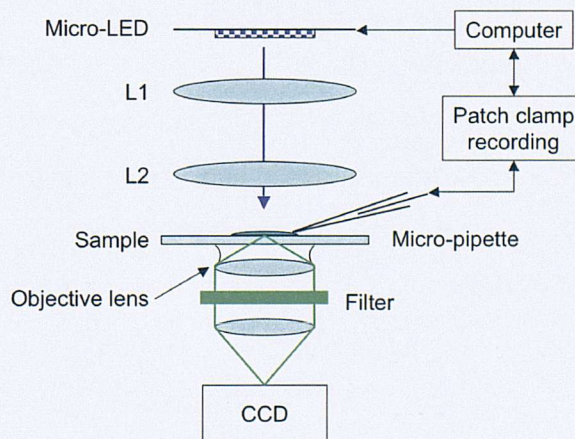


Fig. 7.6. Micro-LED patch clamping setup

In order to stimulate neurons the micro-LED array must be imaged onto the neuron cell using a suitable optical system. This system must provide the required spatial profile and sufficient irradiance for photo-stimulation. Additionally, other factors such as appropriate working distance to accommodate patch-clamping micro-pipettes, perfusion chambers, probes etc. must be taken into account. For the initial instrument a 1:1 4f relay comprising two 50 mm triplet lenses (Sill Optics GmbH S5LPJ2851) was chosen. Although the system is not diffraction limited, the peak to valley aberrations are less than 1.5 waves across the entire field and greater than 90 percent of the light collected from a 17 μm diameter emitter is contained within a 32 μm diameter circle at the image plane. Thus for centre-centre emitter spacings of >32 μm crosstalk from adjacent emitters is low. The working distance of this arrangement is 40 mm allowing good access to cell culture. However the numerical aperture (NA) of the collection system is low (0.14) and given unity magnification the irradiance at the sample will be no greater than 2% of that at the diode chip.

LEDs have an approximately Lambertian emission profile (the light is emitted into a solid angle of 2π steradians and the radiant intensity is proportional to the cosine of the angle relative to the surface normal). In order to collect as much light as possible from the LED then the imaging system should have as large an input NA as possible. For a small Lambertian source the collection efficiency η of a lens having numerical aperture NA is given by $\eta \propto \text{NA}^2$. For example, a lens with a numerical aperture of 0.5 will collect 25% of the total emission from the LED. Progressing through the optical projection system, the Lagrange invariant states that the brightness (power per unit area per unit solid angle) can never be increased beyond that of the object – or rather that collected from the object by the input NA of the system. However, if the image is de-magnified then the output NA will be larger than the input NA (the magnification, M , is the ratio of the input NA to the output NA) and thus because the solid angle of illumination is increased then the absolute irradiance (power per unit area) is increased by $1/M^2$. It is this irradiance (power per unit area) that is important for photo-stimulation. The trade off in this case is that a smaller field of view is covered by the projected image and inevitably the working distance is reduced because of the use of higher NA optics. Likewise the use of micro-lenses at each LED emitter cannot increase the brightness of the source, but they can serve to increase the effective fill factor of the array and hence may be useful in allowing the

smaller fill factor on the LED array itself with resulting benefits in heat generation and dissipation.

Optical power from the LED was measured at the diode and at the sample plane, both for the 4-f imaging system described earlier and for a 40x 0.8NA water dipping objective, which provided with a demagnification M at the sample given by:

$$M = \frac{\text{focal length 1}}{\text{focal length objective}} = 50 \times \frac{40}{180} = 11.1 \quad (6.31)$$

where the focal length of the normal tube lens is 180mm. The power of a single emitter from a blue flip-chip matrix array driven at 3mA was measured 75 μ W at the diode. When imaging the emitter using the 4-f system, irradiance at the sample was measured to be 0.82 μ W, which implies a collection efficiency of about 1.10%. This is lower than the 2% expected from the calculation described earlier and can be attributed to various losses when light propagates through the imaging system and to an emission profile which is not strictly Lambertian. The emitter was also directly imaged onto a CCD camera and its diameter was estimated to be 32 μ m, which resulted in an absolute irradiance of about 1nW. μ m⁻². This irradiance is about an order of magnitude higher than that required for photo-stimulating ChR2 sensitised neurons.

When the water-dipping objective is used at the sample side, irradiance at the sample was measured to be 0.35 μ W over a 3 μ m diameter spot, which corresponds to an irradiance of about 49nW. μ m⁻². The microscope objective one gives more light per unit area over a smaller area, as expected.

2.2.2 Patch-clamping techniques for recording neuron signals

Patch clamping is an established electrophysiological technique that allows for the recording of macroscopic whole-cell or microscopic single channel current flowing across biological membranes through ion channels. The flow of charge carriers across the membrane produces measurable currents, if the transport is not electro-neutral. Both extracellular cell-attached and whole-cell recording techniques are used here together with the LED array, depending on the cell parameter to be investigated. Many different patch-clamping configurations exist [153], but are outside the scope of this thesis.

Extracellular cell-attached recording

Recording of action potentials were first made using extracellular electrodes. A micropipette containing the electrode is filled with an ionic solution, usually sodium chloride, and the tip is brought close to the neuron membrane. The electrode forms a seal on contact with the cell membrane, which is converted to a gigaohm seal by gentle suction. When the electrode is connected to an appropriate amplifier, small currents across the patch of membrane can be recorded [154]. The isolation of a small patch of membrane enables the observation of activity of only a few channels, rather than the thousands that may be active in a whole cell. Thanks to the very high resistance seal, very small currents can be measured. Finally, because this method is non-invasive, long-term experiments can potentially be realised, assuming the cell environment remains constant. This technique is used during the experiments presented in this chapter when verifying neuron excitability without requiring any precise intracellular current or voltage measurements.

Whole-cell recording

This configuration is employed when ion currents of the entire cell are to be recorded. It has the advantage of recording an average response of all the ion channels in the cell membrane and using a micropipette allows modification of the internal environment of the cell, if required. This technique is used in this chapter when recording quantitative intracellular currents, both in current and voltage clamp modes.

Fig. 7.7 shows a schematic of the cell-attached and whole cell patch clamping configurations. In the whole-cell experiment, the micro-pipette breaks into the cell to form a direct electrical contact to the inside of the cell and thus can record an average response of all the ion channels across the whole of the cell membrane.

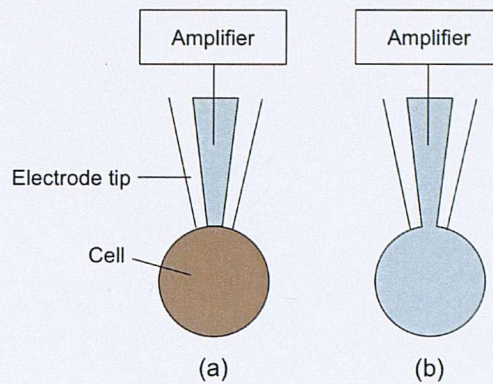


Fig. 7.7. Patch clamping recording configurations represented schematically. (a) Cell-attached and (b) whole cell configurations.

Neuron electro-recording can be operated in current or voltage clamp modes. In current clamp mode, the electrical current passing through the cell membrane is artificially kept constant while stimulating the cell. Voltage changes are then recorded at constant current. This type of recording can be used for both extracellular and intracellular recordings. Typically, changes in voltage of about 0.1 mV to 200 mV are recorded in this manner, and changes in voltage lasting only a fraction of a millisecond can be captured. In voltage clamp mode, the membrane potential is kept constant while stimulating the neuron, typically at -50 to -70 mV. In the meantime, the current of the order of hundreds of pA passing through the membrane is recorded.

2.3. Results

2.3.1 *Photo-stimulation using stripe micro-diodes*

The first step towards stimulating neurons with micro-LEDs was checking that the LED irradiance was sufficient to trigger reliable action potentials in ChR2 photosensitised neurons. A blue stripe μ LED array was first shone onto ChR2 hippocampal neurons. The neurons were obtained from rats at embryonic day 18 and were grown for 12 days in-vitro. The photosensitization was achieved by transfecting the cells to produce ChR2. Responses from single cells were recorded with a standard patch clamping setup (HEKA epc10 double patch clamp amplifier, operating with HEKA Pulse software) [155] in cell attached mode. The cell attached mode provides a quick qualitative overview of the neuron response but the amplitude of the cell response has little quantitative meaning here. Using a micro-LED as the primary stimulation source,

the spatio-temporal resolution enabled stimulation of single cells with sub-cellular resolution.

Fig. 7.8 shows fluorescence images of a ChR2 transfected neuron (the ChR2 is coupled to YFP here) superimposed with several LED stimulation patterns. In Fig. 7.8 (a) the soma is illuminated by single stripe from the blue μ LED. The corresponding graph shows the neuron response to a train of 4 light pulses having durations of 10ms and a frequency of 10Hz. Individual action potentials were accurately and reliably triggered up to a frequency of 40 Hz. The LED was driven using currents of 30 mA, resulting in an irradiance at the sample of $300 \text{ pW}/\mu\text{m}^2$. In contrast, Fig. 7.8 (b)-(d) show the case when the stripe illuminating the soma was turned off, and adjacent stripes illuminating the axon and dendrites were turned on. No reliable action potentials could be achieved compared with somatic excitation, because of the bigger overlap when illuminating the soma. It is also believed that some parts of the soma show higher levels of ChR2 protein expression.

The spontaneous generation of action potentials under somatic excitation was then investigated. The neuron soma was illuminated for 500 ms at various light intensities and the neuron was left to relax down to its initial state for 20 s between two consecutive measurements. First, measurements show an increase in spiking frequency when illumination intensity is increased (Fig. 7.9). The neuron integrates the photo-current before reaching the threshold depolarisation voltage that triggers an action potential. The neuron then regenerates and starts integrating light for the next event. Thus, brighter illumination results in faster integration of the light, which translates into a higher spiking frequency. Secondly, a shift in the frequency of spontaneous events can be observed in all the measurements. Fewer and fewer ChR2 proteins are available in the ground state after each excitation because they remain in the deactivated state for a relatively long period (Fig. 7.5), the cell therefore needs to integrate light for longer before its membrane reaches the threshold potential. This translates into a decrease in the frequency of the spontaneous neuron activity.

It can also be observed that ChR2 neurons offer a very reliable biological system. Indeed the frequencies of the spontaneous spiking patterns are almost identical for the two separate experiments shown in Fig. 7.9 (d) and Fig. 7.9 (e) under the same illumination conditions.

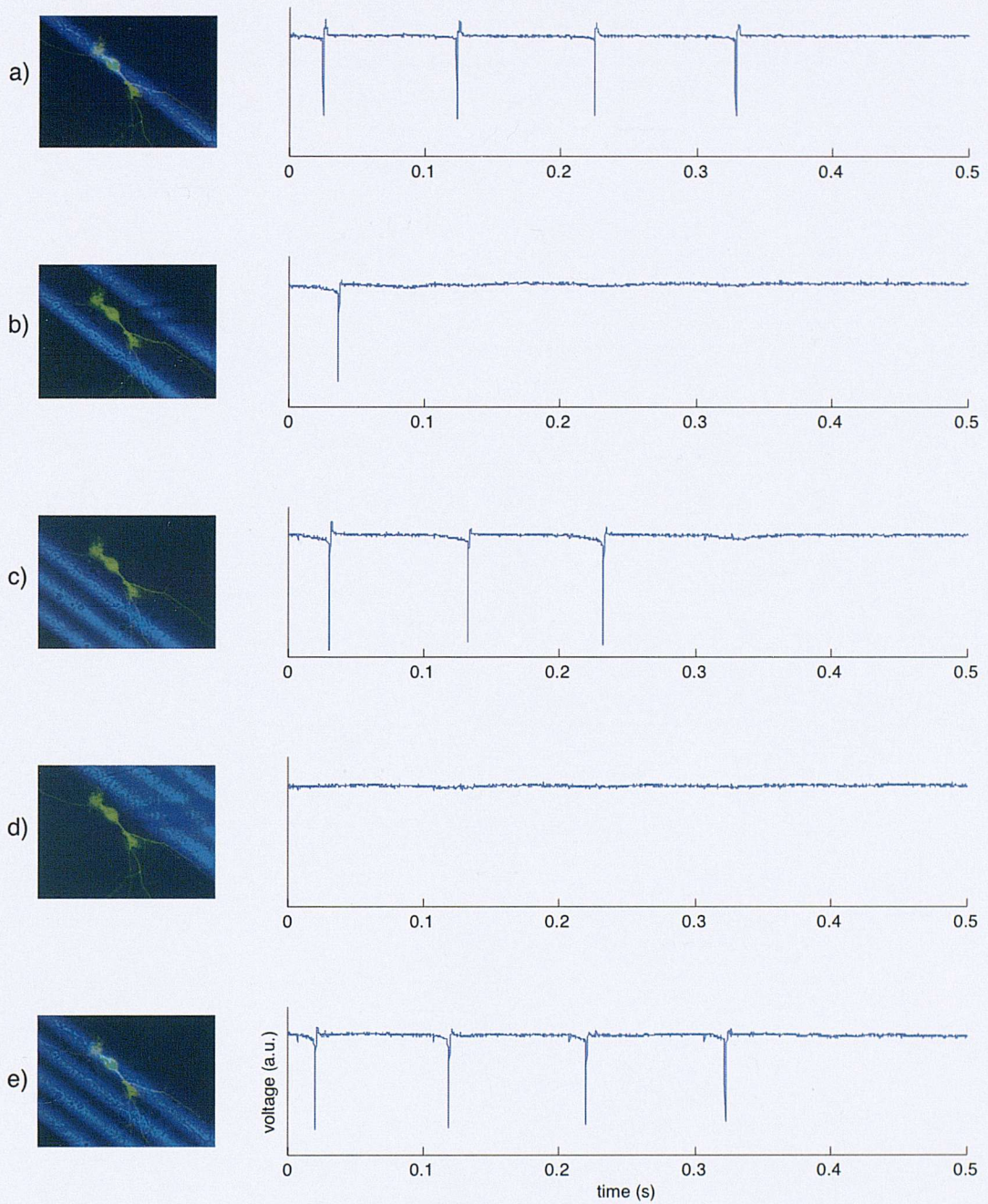


Fig. 7.8. Stimulation of hippocampal neurons using a blue stripe LED array. (a) Somatic pulsed illumination results in corresponding spike train. (b-d) Illumination with neighbouring stripes has various effects on cell depolarization. Pulse train had 4 light pulses having a duration of 10ms and a frequency of 10 Hz.

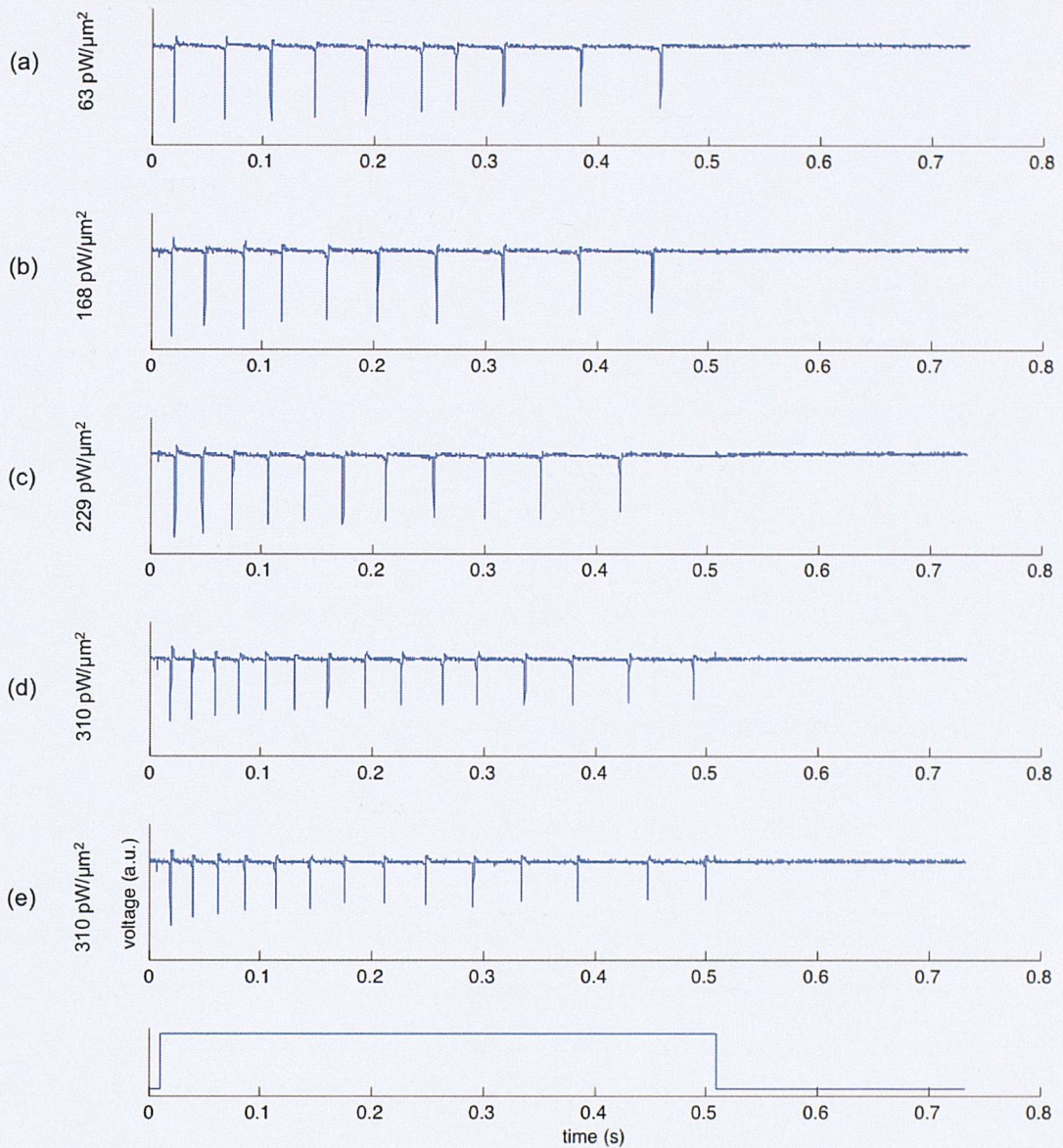


Fig. 7.9. Effect of the illumination intensity on the spontaneous neuron activity. The bottom graph represents the illumination time profile.

Finally, it can be noted that the height of the action potentials decreases with time under an intense and continuous illumination (Fig. 7.9 (d)-(e)). Although unusual, this is well described by introducing a longer recovery time for the sodium channels when modelling the membrane currents involved in the action potential. Indeed opened ChR2 proteins compete with the K^+ channels when repolarising the cell membrane, which results in a less efficient hyperpolarisation and then a slower

recovery for the sodium channels. This turns into a smaller action potential, as less sodium channels become available. A more complete model to describe how the different inwards and outwards ionic currents evolve with time is currently under investigation (N. Grossman, personal communication).

2.3.2 Photo-stimulation using a matrix LED

The illumination system was then modified to offer a true 2D stimulation capability and the stripe LED array was replaced with a flip-chip matrix of blue emitters. Neuron responses were measured by patch clamping after breaking into the cell, which provided quantitative measurements. Fig. 7.10 shows fluorescence images of hippocampal neuron cells transfected with ChR2 marked with YFP and the corresponding voltage and current responses. For the current measurements, the cell membrane potential was clamped at -70mV. LEDs were driven at 3mA per emitter and the neuron was illuminated with a train of 3 pulses having a 10ms duration and at a 10Hz frequency. It can be observed that voltage and current responses are different depending on where the light hits the neuron. A somatic excitation results in a large inwards current, which triggers a single action potential when the cell is first excited (Fig. 7.10 (a)), whereas dendritic excitations have little effect on the cell current, as shown in Fig. 7.10 (b) and (c). However, as the current is proportional to the number of opened ion channels, dendritic excitations add up with the somatic excitation and help triggering more reliable action potentials (Fig. 7.10 (d)). More measurements were used to improve the kinetic model of the ChR2 protein as a main aim of the work being undertaken at the IBE, but the photo-physics of the ChR2 protein is outside the scope of this thesis.

Future experiments will focus on the demagnifying optical system where smaller images of the LED array on the specimen will be used to investigate the mapping the ChR2 protein expression across the neuron. More experiments will also concentrate on axon differentiation during neuron growth under localised blue light excitation.

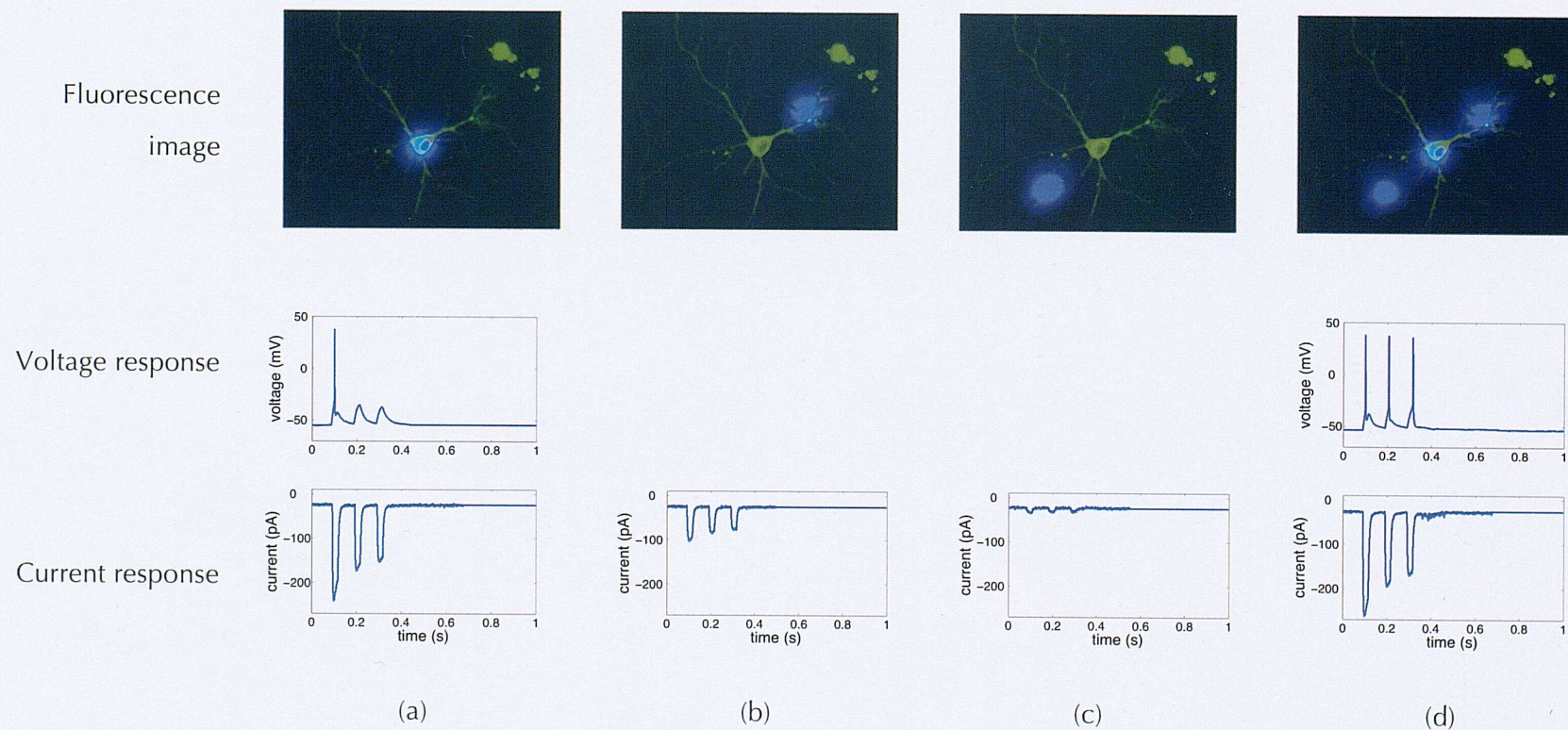


Fig. 7.10. Neuro-stimulation using a matrix micro-LED showing the fluorescence image of a neuron and the corresponding voltage and current responses. (a) Somatic excitation, (b) and (c) dendritic excitation, (d) integrated excitation.

3. Future work

3.1. Multi-electrode recording in a neuron network under multi site illumination

3.1.1 *Multi-electrode array technology (MEA)*

Recently the emergence of multi-electrode array (MEA) technology has provided researchers with multi site recording and even stimulation capabilities [156]. Multi-electrode technology has been developed to study electrophysiological processes at the neuron network level. Although the first MEA system was demonstrated in the 1970's, recent advances in electronic technology have resulted in a revival of this technique. MEA technology is used today extensively to study the dynamics of interactions within neuron networks [157], synaptic plasticity [158], visual perception [159] and effects of pharmacological compounds and putative therapeutics [160]. Fig. 7.11 and Fig. 7.12 show a picture of an MEA plate where cardiac cells have been grown, and the corresponding cardiac activity recorded with a particular electrode.



Fig. 7.11. Picture of an MEA plate with a culture of cardiac cells

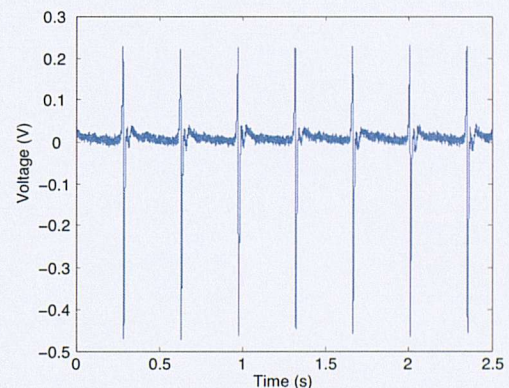


Fig. 7.12. In-vitro electrode recording of cardiac cell activity

MEA systems rely on an MEA plate (Fig. 7.11) connected to an amplifier that filters and amplifies the extra-cellular neuron signals and sends data to the computer. The MEA plate is composed of a matrix of low-impedance electrodes, which are individually sampled at a frequency up to 50 kHz. The present layout enables stimulating and recording responses from different areas within a biological sample. However, MEA systems have currently severe limitations.

- The need to reconcile both electrical stimulation and recording using the same electrodes results in very complex driving electronics, a relative loss of synchronicity between the stimulation and the recording of the neuron signal, and recording artefacts. Indeed, very sensitive recording electrodes get rapidly saturated when electrical excitation is applied in their vicinity, and thus need to be grounded during neuron stimulation.
- As a result of this complexity, the number of stimulation points is limited, currently up to 4 stimulating electrodes in parallel.
- Electrical stimulation has a limited spatial resolution. As the electrical field is transmitted by the cell solution, cells in the vicinity up to 100 microns around the stimulating electrode may be stimulated. Furthermore, stimulation efficiency decays with distance from the electrode, which results in an inhomogeneous stimulation throughout the array.
- It is only possible to stimulate neurons via the electrodes. They cannot be inhibited.

Using micro-structured LED arrays as light sources for stimulating photosensitized neurons should overcome all these issues. First MEA electronics could be much simpler as only the recording capability is needed. Arbitrary stimulation patterns can be shone onto the neuron network and can be modified in real-time in response to the neuron signals. Spatial resolution for neuro-stimulation is ultimately limited by diffraction and neurons can be precisely targeted. And finally, using different illumination wavelengths, along with both cation and anion channel proteins, should enable both excitation and inhibition of the neural activity [129]. This feature will be of particular importance for mimicking real brain activity.

3.1.2 *Experimental setup*

In order to use micro-LED array devices for photo-stimulation and simultaneous electrical recording of nerve cells the system shown schematically in Fig. 7.13 is proposed. The system is constructed around an inverted microscope for sample imaging and alignment as shown in Fig. 7.13. The MEA system is mounted on the stage of this microscope and photo-stimulation is provided by mounting the LED array and projection optics in a trans-illumination position above the stage. Ancillary electronics including the LED driver and MEA instrumentation are controlled by a

computer, which can adjust the stimulation and record the nerve cell response as required. Commercially available MEA recording systems typically consist of a matrix of Indium Tin Oxide electrodes on a glass substrate, coated with Titanium Nitride at the stimulation/recording points. Non-stimulating areas are passivated with silicon nitride. The array is thus fairly transparent to both trans-illumination and epi-illumination light. The inter-electrode spacing is between $100\mu\text{m}$ and $500\mu\text{m}$. Typical diameters of the electrode pad are between $10\mu\text{m}$ and $20\mu\text{m}$, and the electrodes are arranged in an 8×8 array.

Fig. 7.14 shows a 20x picture of an MEA illuminated with a micro-LED array. This MEA consists here in an 8×8 matrix of $20\mu\text{m}$ electrodes separated by $100\mu\text{m}$.

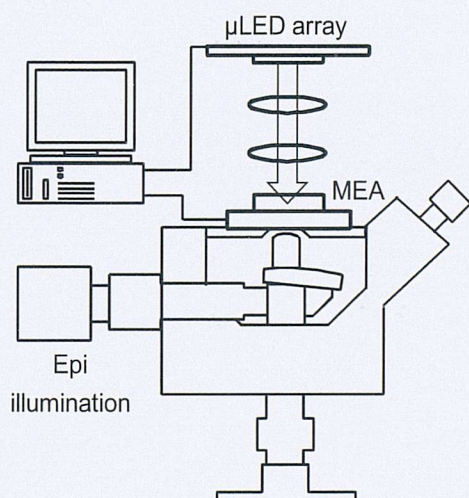


Fig. 7.13. Micro-LEDs photo-stimulation system

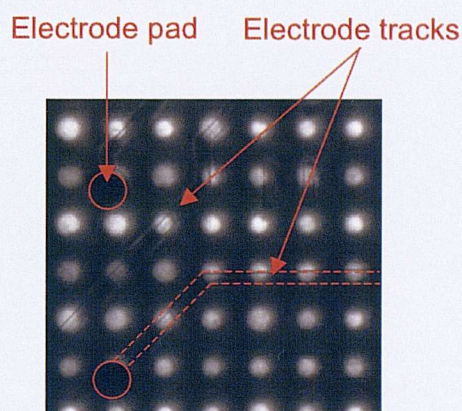


Fig. 7.14. 20x picture of an MEA plate illuminated with an LED matrix

3.1.3 MEA structure

Action potentials have a typical frequency bandwidth between 30 Hz and 3 kHz. In extracellular recording the amplitude of the action potentials is expected to be around few tens of μV peak to peak. In order to be able to detect these signals, a high gain low noise amplifier was used. It has high gain (up to 10,000), very low noise, low DC offset ($\sim 50\mu\text{V}$) and high input impedance ($1\text{ G}\Omega$).

Each microelectrode is connected to a contact pad that lies outside the mini culture chamber. The MEA chip typically sits inside an adapter module that probes the signals from the MEA contact pads. The signal is then amplified, filtered and sent to a data acquisition card (DAQ) that converts it into a digital signal. The digital signal is

then sent to the computer where a dedicated graphical interface is used to analyse and visualize the data.

3.1.4 Graphical interface

A Matlab® graphical interface was designed that allows for a full control of the MEA acquisition parameters, such as the sampling rate, the MEA channels of interest, the signal range and the preview of the current acquisition. Currently, the matrix LED can be controlled only manually by modifying the interface parameters. Whenever real-time online processing is required, additional routines can be easily integrated into the program with minimal modifications of the graphical interface. A screen shot of the graphical interface is presented as Fig. 7.15.

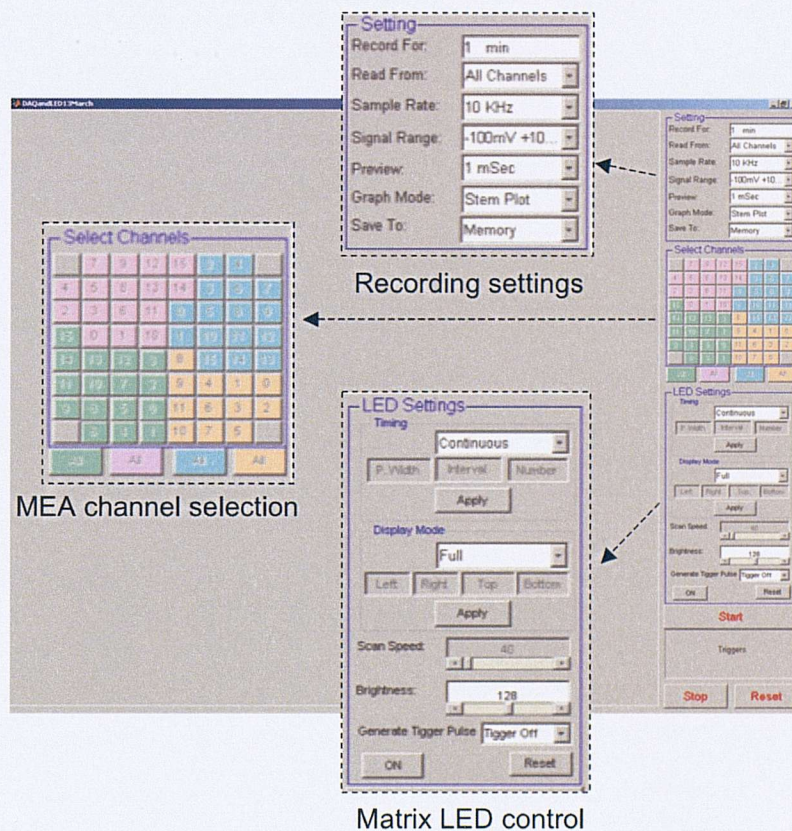


Fig. 7.15. Graphical interface used to control the LED-MEA system

3.1.5 Discussion: Closing the loop

To understand complex neural processing in vertebrates, it is necessary to understand how single neuron/stimulus activity combines to form network level dynamics encompassing sensory inputs, stores memories and control behaviours. Cultured

neuron networks have provided researchers with much of the current understanding of ion channels, receptor molecules and synaptic plasticity that may form the basis of learning and memory [161-164]. The MEA system described above should enable researchers to answer questions not just at a single neuron level but at the network level, by recording signals from up to 64 electrodes and by stimulating/inhibiting photosensitised neurons in up to 64x64 sites. For instance, integrating the MEA system into a real-time sensory-motor loop should enable the neuron network to be used as the “brain” of a robot [165, 166] and would bring in-vitro networks closer to the kind of neural processing that nervous systems evolved for, that is, to take in continuous sensory information, processing it and exhibiting adaptive behaviour continuously. This approach of embodied cultured networks might be used to address how activity shapes neural development and the bases of distributed neural activity for sensory processing, memory formation and behavioural control. Using the current interface of the LED-MEA system, it should be straightforward to integrate routines for on-line processing that will analyse neuron responses in real-time and modify the stimulation pattern accordingly.

3.2. Long term application of GaN LED arrays: the retinal prosthetics project

A number of research groups have recently developed electrical implants that can be attached directly to the retina in order to restore some limited vision to people suffering from retinal degeneration [167-170]. There are currently two kinds of retinal implants; sub-retinal and epi-retinal implants. Both types rely on a physical pixellated sensor penetrating into the retina and special surgical techniques have been developed for implanting the sensors into the eye. However, both techniques restore vision with very low spatial resolution due to the limited number of pixels of the sensors. Furthermore, questions remain about the long-term stability and biocompatibility of implant material. Here, a new concept for non-invasive retinal prosthetics is introduced where the retinal ganglion cells are rendered photo-sensitive after expressing ChR2 and a blue pattern is then projected onto the retina using a matrix of blue micro-LEDs. This work is one of the main thrusts of the IBE group, but shows particularly how the compact and programmable nature of these micro-LED devices can be used to their maximum advantage.

3.2.1 Basics of vision: the visual system

The visual information entering the eye is focused by the optic components of the eye (the cornea and the lens) onto the 130 million photoreceptors of the outermost neuroepithelium layer of the retina at the back of the eye. This sensory layer transforms local optical images into chemical and thus electrical signals. These signals activate a complex circuit of retinal neurons and visual information is compressed into electrical signals carried by 1.2 million ganglion cells, whose axons form the optic nerve. The optic nerve then transmits visual information to the visual cortex.

The optical image is centred on the fovea, which is a 1.5 mm diameter region in the retina with the largest density of receptors and thus has the greatest visual acuity. The fovea is also located on the optic axis for minimal image distortions. About 4 mm from the fovea toward the nose is located the optic disk, where the nerve fibres and retinal blood vessels pierce the retina. Photoreceptors are absent from this region, which results in a *blind spot* in the visual field.

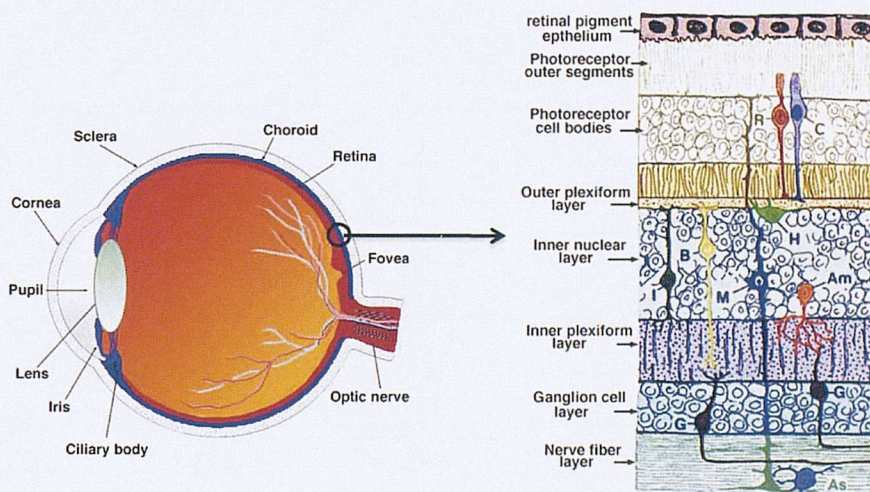


Fig. 7.16. Structures of the eye and retina (from [171])

3.2.2 Retinal dystrophies

Blindness can result when any of the steps in the optical pathway (optics, retina, optic nerve, visual cortex, other cortical area) sustain damage. In western countries, retinal dystrophies, such as Age related Macular Degeneration (AMD) and Retinitis Pigmentosa (RP) account for most of the eye disorders leading to blindness [172].

Age related Macular Degeneration

AMD is the main cause of blindness among adults in the western world [173] and can be defined as abnormal aging of the retinal pigment epithelium (RPE) layer. The disease evolves from lesions at the RPE basement in an early stage into the atrophy of the central RPE cells that causes death of the macular photoreceptors, which results in vision loss. To date, there is no effective treatment for all types of AMD.

Retinitis Pigmentosa (RP)

Retinitis Pigmentosa is a generic name for several hereditary retinal disorders in which abnormalities of the photoreceptors lead to progressive vision loss. It is a leading cause of blindness in younger people and an estimated 1.5 million people are affected. As the disease evolves, patients develop tunnel vision due to photoreceptor apoptosis and eventually lose central vision as well [174]. There is no effective treatment for RP, although several nutritional approaches can help slowing down some forms of the diseases.

For both these retina dystrophies, photoreceptors degrade quickly, whereas ganglion cells and most of the neural circuitry remains in general usable, which suggested the new approach for the retina prosthetics.

3.2.3 Current vision prosthetic technologies

Current visual prosthetics technologies rely on direct electrical stimulation of neurons along the visual pathway, including retinal stimulation, optic nerve stimulation and direct visual cortex stimulation. Due to the complexity of the biological processing occurring between the photoreceptor cells and the brain, the closer the excitation to the photoreceptors, the less bio-mimetic processing is needed in order to present appropriate data to the visual cortex. The most favoured approach to date for visual prosthetics is then implanting an intraocular array of electrodes at the retina.

The retinal implants exist mainly in two forms, namely sub-retinal and epi-retinal implants. Sub-retinal implants rely on an array of photodiodes, which are placed underneath the retinal layer in an attempt to replace the dead photosensitive retina structure [168, 175]. Epi-retinal chips are positioned at the surface of the retina in order to directly stimulate retinal ganglion cells [169, 176]. In this case, additional image processing is required because the initial photoreceptor processing stage is

bypassed. These technologies have been successfully demonstrated to provide patients with some sense of visual perception, but suffer from severe drawbacks:

- Power consumption is a significant issue because introducing power cables directly into the eyeball is not a sustainable solution. Implants currently rely on an energy signal, such as a laser beam or a RF signal, coupled to power the photo-sensor circuitry
- The implants contain invasive electrodes used to inject current into the ganglion cells, which was shown to cause further degradation of the retinal tissue.
- Implants have a limited number of pixels and are difficult to position with high accuracy.

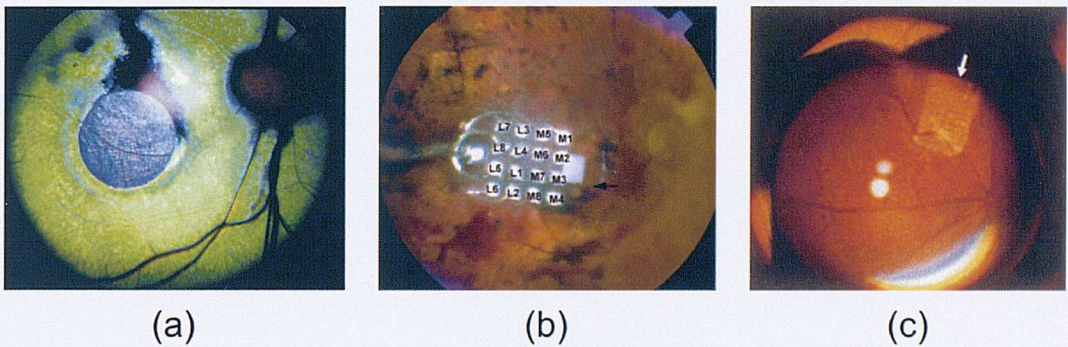


Fig. 7.17. Current approaches in retinal prosthetics: (a)-(b) Sub-retinal prosthetics by Chow [175] and Zrenner [167] and (c) 16 electrode epi-retinal implant by Humayun [176]

3.2.4 LED-based retina prosthetics

In order to overcome these problems, a completely different approach for retinal prostheses consists of transforming communication retinal neurons into photosensitive cells and photo-stimulating them directly. Stimulating the retinal neurons with light instead of electricity enables non-invasive prostheses with superior temporal and spatial resolution, flexibility, and upgradeability. The photonic retinal prosthetics is shown schematically in Fig. 7.18. The device is composed of a pixellated detector such as a CMOS camera to grab an image of the scene, a processing unit that process the image and controls the stimulating pattern for the LED system, an LED system where the processed image is displayed, and appropriate optics (not shown in Fig. 7.18) to project the LED pattern onto the photosensitised retinal ganglion cell layer.

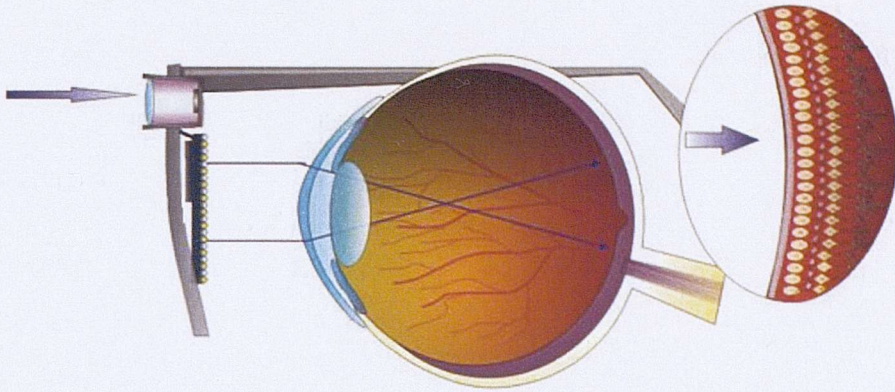


Fig. 7.18. Retinal prosthetic system: The outward scene is captured with a camera. Images are processed and an array of light sources is driven to illuminate the resulting two-dimensional pattern. The pattern is focused by the eye on to the photosensitized residual retinal ganglion cells that send the information to the visual cortex (adapted from [177]).

ChR2 is a promising candidate for photosensitising ganglion cells. Indeed, in order to transmit a reliable image to the visual cortex, activated neurons should only respond to a bright illumination pattern, but not the blue background light inherently present in daylight. The high threshold of the ChR2 protein ensures that the protein remains in the close state for most of the time. Furthermore, ChR2 is proven to exhibit a fast temporal response, which makes it suitable for video rate image projection.

3.2.5 *Challenges*

Several technological and biological challenges remain for LED-based retinal prosthetics:

- Biological vectors, such as inactivated and modified viruses, should be developed to target and transfect only retinal ganglion cells with ChR2, without photo-sensitising the whole central nervous system [152, 178].
- LED technology must be improved to provide uniform illumination, eliminate pixel crosstalk, and lower the power consumption.
- The whole projection system including the driving electronics should be packaged in a compact way to be head-mounted. Integration of CMOS driving circuitry with direct flip-chip mounted micro-LED arrays would prove useful in this respect.

- Integrated projection optics should be designed. Wedge optics [179] for instance could be used to interface the LED array with the eye.
- The ethical question of transfecting a human-body with a plant gene must be addressed.

4. Summary

The high brightness of GaN micro-LEDs enables new applications in neurophysiology where lasers were previously used together with beam scanning units. Photolysis of caged-fluorescein in gel was achieved using a UV micro-LED, and could be extended to caged glutamate release in the neuron vicinity assuming significant improvements in the UV micro-LED emission power and uniformity. Blue emitting micro-arrays were then successfully applied to photosensitised neuron excitation in a patch-clamping recording system. For the first time, multi-site excitations with high temporal and spatial resolution were demonstrated in a compact and easy to use setup. LED arrays proved to be a very reliable solution for stimulation of Channelrhodopsin-2 expressing neurons. This opened up the possibility of high-resolution stimulation and recording of neuron networks by coupling the LED array with multi-site MEA recording. A new system was built. Further experiments will concentrate on using patterned excitations to study connectivity and plasticity of neuron networks. Finally, a long-term project to implement LED-based visual prosthetics was described that will rely on photo-sensitization of the retinal ganglion cells together with LED patterned excitation to trigger neuron responses.

Chapter 8 Conclusion and future work

The goal of this thesis was to demonstrate innovative applications for micro-structured Gallium-Nitride light emitting diodes, which are being developed and fabricated at the University of Strathclyde by a joint UK research council funded Basic Technology Grant. These devices can provide both high brightness illumination and spatial resolution and two main applications are investigated in this thesis: first as a way of achieving optical sectioning in a fluorescence confocal microscope with widefield detection and second as a primary illumination source for neuron photo-stimulation. The process of obtaining sectioned images in a confocal microscope is explained in chapter 2 and several techniques to achieve optical sectioning in a widefield imaging system are reviewed. The advantages of these approaches in terms of acquisition speed and instrumentation ease are described and shown to be a driving force for the use of LED micro-arrays in this application area.

Engineering considerations for designing and driving LED micro-arrays are exposed in chapter 3. The Basic Technology research program started with the design and fabrication of a top-emitting 64x64 blue LED array, which can be driven using a multiplexed matrix-addressing scheme. The first device was characterised and severe defects in the emission uniformity were found due to the electrical resistance of the n-mesa structure increasing along its length. This resulted in the addition of a conductive stripe of metal along the mesa that improved the emission uniformity dramatically. Heat extraction and external quantum efficiency were then improved by using the flip-chip technology. The flip-chip device was characterised to sustain a very high injection current density and had a very high brightness emission. Driving electronics were designed to display arbitrary binary patterns on a microsecond timescale. By sinking a constant current across a column of emitters at a time, non-uniformities could be corrected and reliable emission was achieved. Binary images in excess of 400 frames per second could be displayed when raster-scanning columns. A one-dimensional LED array consisting on 120 stripe emitters was also investigated, for which a driver board was designed and manufactured, allowing the display of arbitrary line patterns at in excess of 50,000 frames per seconds.

In chapter 4, the technique of grid-projection structured illumination microscopy was presented as a way of obtaining confocal-like images in a widefield microscope with devices such as the line LED device described in chapter 3. The sectioning imaging process was explained and performance of the structured illumination system in presence of photon noise was derived. The noise was shown to be strongly dependent of the intensity of the out-of-focus object and is modulated at three times the illumination frequency when an in-focus object is present. This noise anisotropy is only really apparent when the contrast of the projected grid pattern is high and may need to be taken into account when quantitative measurements are to be obtained from optically sectioned images. However it is shown that a ghost image corresponding to the square root of the out-of-focus image can be seen, which could be a significant issue in this kind of microscope. A demonstration system was constructed with the LED stripe array placed in a critical illumination configuration in a microscope operating in fluorescence and grid-projection structured illumination microscopy was achieved with no moving parts. The grid pattern was scanned electronically, which has clear advantages in terms of photo-bleaching, compactness and ease of use, although line artefacts can be observed in the sectioned images due to emission-non-uniformities.

A slit-scanning microscope was implemented in chapter 5 using the same microscope setup where the micro-stripe LED illuminates the specimen a line by line. Several post-processing detection schemes were described to obtain a confocal effect, either by implementing a set of virtual confocal slits in software or by rejecting the out-of-focus blur at a pixel level. The performance of this system was measured and compared well with the theory. Instead of scanning a single line pattern onto the sample, the micro-LED solution made it possible to scan multiple lines at the same time, at the expense of decreasing the sectioning strength of the microscope and introducing line artefacts in the sectioned images due to non-uniform out-of-focus blur when high fill-factor line patterns are used. The work on removing these artefacts led to a new background subtraction technique. Under line illumination and detection, the out-of-focus light that remains in the confocal image is estimated from the signal in adjacent positions in the widefield image and is subtracted. This technique showed a dramatic improvement of the out-of-focus light rejection in a single slit confocal microscope and could be readily applied to existing commercially available systems

with minor system modifications. Background subtraction was successfully applied when scanning multi-line patterns and allowed for correction of the line artefacts in the sectioned images. Acquisition speed was tremendously increased without sacrificing confocality anymore and superior optical sectioning was experimentally demonstrated. For the first time the solid-state line-scanning microscope makes it possible to adapt the imaging process and optical sectioning to the specimen to be imaged.

In chapter 6, the theory of improving optical sectioning by subtracting the out-of-focus blur in the line geometry was presented. The imaging process was described and an analytical expression for the intensity response to defocus was derived. The line subtraction confocal microscope was shown to exhibit an optical sectioning dramatically improved compared to the normal line scanning system and even marginally improved over the point-scanning confocal microscope, which agreed with the experimental measurements. The theory of background subtraction under multi-line illumination was presented and showed a further improvement in the confocality of the line system. The optical sectioning of the multi-line system was shown to converge towards the confocal capability of the structured illumination system when illuminating the specimen with a high fill-factor pattern. This theory also led to the design of an improved double-sided spinning disk microscope system, where out-of-focus light is partly recorded before subtraction. This disk should allow for high frame rate confocal imaging with improved axial resolution, although disk manufacturing might prove challenging.

Finally, applications of LED arrays to neuro-biology were presented in chapter 7. UV micro-stripe arrays were first applied to flash photolysis of caged fluorescein molecules. Under patterned UV illumination caged fluorophores could be released and fluorescence could be excited. UV micro-arrays are therefore promising candidates for neuro-transmitter uncaging where spatially selective excitation of neuro-activity is required. Blue micro-arrays were then applied to direct photostimulation of neurons. These cells were rendered sensitive to blue light after transfection with a direct ion channel membrane protein, ChannelRhodopsin-2. Single neuron activity was recorded by patch-clamping and was shown to be dependent on the stimulation pattern. A new tool for photo stimulation of neuron networks was presented where neurons are excited using a blue LED matrix and neuron activity is

recorded using a multi-electrode array. This system should enable the study of neuron connectivity and plasticity when embedded in a network. Finally, a retinal prosthesis is proposed based on the ChR2 photo-sensitisation of the retina ganglion cells and the projection of bright LED patterns onto the retina. This retinal prosthesis should be non-invasive and safe, providing the ChR2 transfection can be precisely targeted to solely the ganglion cells.

Future work with these LED micro-arrays should first focus on device improvements. For the matrix devices, CMOS driving electronics integrated at the backplane of the devices and should allow for truly independent control of each LED in the array while reducing the number of data connections to only 4 (2 data lines and 2 power lines). Smaller emitters and micro-lenses can be engineered to increase the light collection and effective fill-factor while radically reducing the heat that is generated by the micro-emitters. For line devices, flip-chipping the stripe LED array and depositing thicker diode contacts could dramatically improve current spreading. Again CMOS backplane drive electronics should also allow larger arrays to be driven using fewer connections.

New microscopy applications will result from the miniaturisation via the CMOS backplane. First a hand-held confocal microscope should be easily implemented using a LED array for illumination and a simple CMOS camera for detection. This could make confocal microscopes cheap and easy to use for skin cancer detection. Assuming dedicated thermal engineering, LED arrays could also be integrated into confocal endoscopes. It can also be noted that with UV LEDs (which still need to be improved in terms of efficiency), autofluorescence imaging applications will become possible.

On the photo-stimulation side, future work will continue on creating a simple neuron-computer interface that allows for mapping neuron connections and studying their plasticity in a neuron network. Long-term experiments can be envisaged where the neuron network adapt itself to external stimuli in order to reproduce the learning process in-vitro. Axon guidance via long-term light stimulation will also be investigated during neuron differentiation.

In conclusion, this thesis investigated the applications of micro-structured GaN light emitting diodes to microscopy and neurophysiology. The LED structure allows for blue and UV intense pattern illumination, which was used to achieve optical

sectioning microscopy with no moving parts and stimulation of photosensitised neurons with spatial resolution. Although the technology can still be greatly improved, the numerous advantages of the micro-LED displays will assure their wider application in bio-photonics.

References

1. M. Minsky, "Microscopy apparatus," (US, 1961).
2. T. Wilson, *Confocal Microscopy* (Academic Press, San Diego, 1990).
3. J. B. Pawley, *Handbook of Biological Confocal Microscopy* (Springer, 2006).
4. D. M. Maurice, "A Scanning Slit Optical Microscope," *Investigative Ophthalmology Visual Science* **13**, 1033-1037 (1974).
5. T. Wilson, and A. R. Carlini, "Imaging in scanning microscopes with slit-shaped detectors," *Journal of Microscopy* **160**, 115-139 (1990).
6. B. R. Masters, "Real-time scanning slit confocal microscopy of the in vivo human cornea," *Appl. Optics* **33**, 695 (1994).
7. M. Petran, M. Hadravsk, M. D. Egger, and R. Galambos, "Tandem-Scanning Reflected-Light Microscope," *J. Opt. Soc. Am.* **58**, 661 (1968).
8. T. Wilson, M. A. A. Neil, and R. Juskaitis, "Confocal microscopy apparatus and method," (Isis Innovation Limited, US, 2004).
9. J. Huisken, J. Swoger, F. Del Bene, J. Wittbrodt, and E. H. K. Stelzer, "Optical sectioning deep inside live embryos by selective plane illumination microscopy," *Science* **305**, 1007-1009 (2004).
10. H.-U. Dodt, U. Leischner, A. Schierloh, N. Jahrling, C. P. Mauch, K. Deininger, J. M. Deussing, M. Eder, W. Zieglgansberger, and K. Becker, "Ultramicroscopy: three-dimensional visualization of neuronal networks in the whole mouse brain," *Nat Meth* **4**, 331-336 (2007).
11. P. A. Temple, "Total internal reflection microscopy: a surface inspection technique," *Appl. Opt.* **20**, 2656 (1981).
12. D. Axelrod, "Total Internal Reflection Fluorescence Microscopy in Cell Biology," *Traffic* **2**, 764-774 (2001).
13. J. Schmoranzner, M. Goulian, D. Axelrod, and S. M. Simon, "Imaging Constitutive Exocytosis with Total Internal Reflection Fluorescence Microscopy," *The Journal of Cell Biology* **149**, 23-32 (2000).
14. M. A. A. Neil, R. Juskaitis, and T. Wilson, "Method of obtaining optical sectioning by using structured light in a conventional microscope," *Opt. Lett.* **22**, 1905-1907 (1997).
15. M. A. A. Neil, R. Juskaitis, and T. Wilson, "Real time 3D fluorescence microscopy by two beam interference illumination," *Opt. Commun.* **153**, 1-4 (1998).
16. M. A. A. Neil, A. Squire, R. Juskaitis, P. I. H. Bastiaens, and T. Wilson, "Wide-field optically sectioning fluorescence microscopy with laser illumination," *J. Microsc.-Oxf.* **197**, 1-4 (2000).
17. D. Karadaglic, and T. Wilson, "Image formation in structured illumination wide-field fluorescence microscopy," *Micron* **In Press, Corrected Proof** (2008).
18. T. Wilson, R. Juskaitis, M. A. A. Neil, and M. Kozubek, "Confocal microscopy by aperture correlation," *Opt. Lett.* **21**, 1879-1881 (1996).
19. C. Ventalon, and J. Mertz, "Quasi-confocal fluorescence sectioning with dynamic speckle illumination," *Opt. Lett.* **30**, 3350-3352 (2005).
20. C. Ventalon, and J. Mertz, "Dynamic speckle illumination microscopy with translated versus randomized speckle patterns," *Opt. Express* **14**, 7198-7209 (2006).
21. C. Ventalon, R. Heintzmann, and J. Mertz, "Dynamic speckle illumination microscopy with wavelet prefiltering," *Opt. Lett.* **32**, 1417-1419 (2007).

22. D. S. Nakamura, "- Introduction to Nitride Semiconductor Blue Lasers and Light Emitting Diodes," (2000).
23. C. W. Jeon, H. W. Choi, and M. D. Dawson, "A novel fabrication method for a 64x64 matrix-addressable GaN- based micro-LED array," *Phys. Status Solidi A-Appl. Res.* **200**, 79-82 (2003).
24. C. W. Jeon, H. W. Choi, and M. D. Dawson, "Fabrication of matrix-addressable InGaN-based microdisplays of high array density," *IEEE Photonics Technol. Lett.* **15**, 1516-1518 (2003).
25. H. W. Choi, C. W. Jeon, and M. D. Dawson, "High-resolution 128 x 96 nitride microdisplay," **25**, 279 (2004).
26. C. W. Jeon, H. W. Choi, E. R. Gu, and M. D. Dawson, "High-density matrix-addressable AlInGaN-Based 368-nm microarray light-emitting diodes," *IEEE Photonics Technol. Lett.* **16**, 2421-2423 (2004).
27. H. X. Zhang, E. Gu, C. W. Jeon, Z. Gong, M. D. Dawson, M. A. A. Neil, and P. M. W. French, "Microstripe-array InGaN light-emitting diodes with individually addressable elements," *Photonics Technology Letters, IEEE* **18**, 1681-1683 (2006).
28. Z. Gong, H. X. Zhang, E. Gu, C. Griffin, M. D. Dawson, V. Poher, G. Kennedy, P. M. W. French, and M. A. A. Neil, "Matrix-Addressable Micropixelated InGaN Light-Emitting Diodes With Uniform Emission and Increased Light Output," *Electron Devices, IEEE Transactions* **54**, 2650-2658 (2007).
29. M. Born, and E. Wolf, "Principles of Optics: electromagnetic theory of propagation, interference and diffraction of light," (1999).
30. J. W. Goodman, *Introduction to Fourier Optics* (McGraw Hill, 1996).
31. H. H. Hopkins, "The Frequency Response of a Defocused Optical System," *Proceedings of the Royal Society of London. Series A* **231**, 91-103 (1955).
32. P. A. Stokseth, "Properties of a Defocused Optical System," *Journal of the Optical Society of America* **59**, 1314 (1969).
33. C. J. R. Sheppard, and T. Wilson, "Depth of field in the scanning microscope," *Opt. Lett.* **3**, 115 (1978).
34. B. R. Frieden, "Optical transfer of the three-dimensional object," *J. Opt. Soc. Am.* **57**, 56 (1967).
35. C. J. R. Sheppard, and M. Gu, "Approximation to the three-dimensional optical transfer function," *J. Opt. Soc. Am. A* **8**, 692 (1991).
36. J. Philip, "Optical transfer function in three dimensions for a large numerical aperture," *J. Mod. Opt.* **46**, 1031-1042 (1999).
37. P. Davidovits, and M. D. Egger, "Scanning laser microscope for biological investigations," *Appl. Opt.* **10**, 1615-1619 (1971).
38. G. J. Brakenhoff, J. S. Binnerts, and P. Barends, "High resolution confocal scanning light microscopy (CSLM) as applied to living biological specimens.," *SEM SERIES*, 1-138 (1981).
39. T. Wilson, and A. R. Carlini, "Size of the detector in confocal imaging systems," *Opt. Lett.* **12**, 227 (1987).
40. S. Kimura, and C. Munakata, "Dependence of 3-D optical transfer functions on the pinhole radius in a fluorescent confocal optical microscope," *Appl. Opt.* **29**, 3007 (1990).
41. J. R. Lakowicz, *Principles of Fluorescence Spectroscopy* (SPRINGER, 2006).
42. G. G. Stokes, "On the Change of Refrangibility of Light," *Philosophical transactions of the Royal Society of London* **142**, 463 (1852).

43. S. J. Strickler, and R. A. Berg, "Relationship between Absorption Intensity and Fluorescence Lifetime of Molecules," *The Journal of Chemical Physics* **37**, 814-822 (1962).
44. A. Einstein, "Zur Quantentheorie der Strahlung," *Physik Zeitschr* **18**, 121-128 (1917).
45. R. Sanders, A. Draaijer, H. C. Gerritsen, P. M. Houpt, and Y. K. Levine, "Quantitative pH Imaging in Cells Using Confocal Fluorescence Lifetime Imaging Microscopy," *Analytical Biochemistry* **227**, 302-308 (1995).
46. H. H. Szmazinski, and J. J. R. Lakowicz, "Optical measurements of pH using fluorescence lifetimes and phase-modulation fluorometry," *Analytical chemistry* **65**, 1668-1674 (1993).
47. K. Dowling, M. J. Dayel, M. J. Lever, P. M. W. French, J. D. Hares, and A. K. L. Dymoke-Bradshaw, "Fluorescence lifetime imaging with picosecond resolution for biomedical applications," *Opt. Lett.* **23**, 810-812 (1998).
48. F. Jähnig, "Structural order of lipids and proteins in membranes: evaluation of fluorescence anisotropy data," *Proc Natl Acad Sci USA* **76**, 6361-6365 (1979).
49. D. Axelrod, "Carbocyanine dye orientation in red cell membrane studied by microscopic fluorescence polarization," *Biophys. J.* **26**, 557-573 (1979).
50. J. Siegel, K. Suhling, S. Leveque-Fort, S. E. D. Webb, D. M. Davis, D. Phillips, Y. Sabharwal, and P. M. W. French, "Wide-field time-resolved fluorescence anisotropy imaging (TR-FAIM): Imaging the rotational mobility of a fluorophore," *Review of Scientific Instruments* **74**, 182-192 (2003).
51. C. J. R. Sheppard, and X. Q. Mao, "Confocal Microscopes with Slit Apertures," *J. Mod. Opt.* **35**, 1169-1185 (1988).
52. S. Kimura, and C. Munakata, "Three-dimensional optical transfer function for the fluorescent scanning optical microscope with a slit," *Appl. Optics* **29**, 1004-1007 (1990).
53. D. R. Sandison, and W. W. Webb, "Background rejection and signal-to-noise optimization in the confocal and alternative fluorescence microscopes," *Appl. Opt.* **33**, 603 (1994).
54. K. B. Im, S. Han, H. Park, D. Kim, and B. M. Kim, "Simple high-speed confocal line-scanning microscope," *Opt. Express* **13**, 5151-5156 (2005).
55. R. Wolleschensky, B. Zimmermann, and M. Kempe, "High-speed confocal fluorescence imaging with a novel line scanning microscope," *Journal of Biomedical Optics* **11**, 064011-064014 (2006).
56. D. T. Fewer, S. J. Hewlett, E. M. McCabe, and J. Hegarty, "Direct-view microscopy: Experimental investigation of the dependence of the optical sectioning characteristics on pinhole-array configuration," *J. Microsc.-Oxf.* **187**, 54-61 (1997).
57. S. J. Hewlett, D. T. Fewer, and E. M. McCabe, "Influence of source coherence and aperture distribution on the imaging properties in direct-view microscopy," *J. Opt. Soc. Am. A* **14**, 1066-1075 (1997).
58. E. Wang, C. M. Babbey, and K. W. Dunn, "Performance comparison between the high-speed Yokogawa spinning disc confocal system and single-point scanning confocal systems," *Journal of Microscopy* **218**, 148-159 (2005).
59. J. M. Murray, P. L. Appleton, J. R. Swedlow, and J. C. Waters, "Evaluating performance in three-dimensional fluorescence microscopy," *Journal of Microscopy* **228**, 390-405 (2007).
60. R. Juskaitis, T. Wilson, M. A. A. Neil, and M. Kozubek, "Efficient real-time confocal microscopy with white light sources," **383**, 804-806 (1996).

61. P. J. Verveer, Q. S. Hanley, P. W. Verbeek, L. J. Van Vliet, and T. M. Jovin, "Theory of confocal fluorescence imaging in the programmable array microscope (PAM)," *J. Microsc.-Oxf.* **189**, 192-198 (1998).
62. Q. S. Hanley, P. J. Verveer, and T. M. Jovin, "Optical sectioning fluorescence spectroscopy in a programmable array microscope," *Appl. Spectrosc.* **52**, 783-789 (1998).
63. R. Heintzmann, Q. S. Hanley, D. Arndt-Jovin, and T. M. Jovin, "A dual path programmable array microscope (PAM): simultaneous acquisition of conjugate and non-conjugate images," *J. Microsc.-Oxf.* **204**, 119-135 (2001).
64. S.-h. Jiang, and J. G. Walker, "Experimental confirmation of non-scanning fluorescence confocal microscopy using speckle illumination," *Opt. Commun.* **238**, 1-12 (2004).
65. M. G. Somekh, C. W. See, and J. Goh, "Wide field amplitude and phase confocal microscope with speckle illumination," *Opt. Commun.* **174**, 75-80 (2000).
66. P. J. Verveer, J. Swoger, F. Pampaloni, K. Greger, M. Marcello, and E. H. K. Stelzer, "High-resolution three-dimensional imaging of large specimens with light sheet-based microscopy," *Nat Meth* **4**, 311-313 (2007).
67. M. G. L. Gustafsson, "Surpassing the lateral resolution limit by a factor of two using structured illumination microscopy," *J. Microsc.-Oxf.* **198**, 82-87 (2000).
68. M. G. L. Gustafsson, L. Shao, P. M. Carlton, C. J. R. Wang, I. N. Golubovskaya, W. Z. Cande, D. A. Agard, and J. W. Sedat, "Three-dimensional Resolution Doubling in Widefield Fluorescence Microscopy by Structured Illumination," *Biophysical Journal*, biophysj.107.120345 (2008).
69. M. G. L. Gustafsson, "Nonlinear structured-illumination microscopy: Wide-field fluorescence imaging with theoretically unlimited resolution," *Proceedings of the National Academy of Sciences* **102**, 13081-13086 (2005).
70. B. Bailey, D. L. Farkas, D. L. Taylor, and F. Lanni, "Enhancement of axial resolution in fluorescence microscopy by standing-wave excitation," *Nature* **366**, 44-48 (1993).
71. B. B. Albrecht, A. V. A. V. Failla, A. A. Schweitzer, and C. C. Cremer, "Spatially modulated illumination microscopy allows axial distance resolution in the nanometer range," *Applied optics* **41**, 80-87 (2002).
72. F. Helmchen, and W. Denk, "Deep tissue two-photon microscopy," *Nat Meth* **2**, 932-940 (2005).
73. M. Göppert-Mayer, "Über Elementarakte mit zwei Quantensprüngen," *Annalen der Physik* **401**, 273 (1931).
74. A. Nimmerjahn, F. Kirchhoff, J. N. D. Kerr, and F. Helmchen, "Sulforhodamine 101 as a specific marker of astroglia in the neocortex in vivo," *Nat Meth* **1**, 31-37 (2004).
75. K. Svoboda, "In vivo dendritic calcium dynamics in neocortical pyramidal neurons," *Nature* **385**, 161 (1997).
76. J. Bewersdorf, and S. W. Hell, "Multifocal multiphoton microscopy," *Opt. Lett.* **23**, 655 (1998).
77. K. Fujita, O. Nakamura, T. Kaneko, M. Oyamada, T. Takamatsu, and S. Kawata, "Confocal multipoint multiphoton excitation microscope with microlens and pinhole arrays," *Opt. Commun.* **174**, 7-12 (2000).
78. A. D. Elder, J. H. Frank, J. Swartling, X. Dai, and C. F. Kaminski, "Calibration of a wide-field frequency-domain fluorescence lifetime microscopy system using light emitting diodes as light sources," *Journal of Microscopy* **224**, 166-180 (2006).

79. C. Moser, T. Mayr, and I. Klimant, "Filter cubes with built-in ultrabright light-emitting diodes as exchangeable excitation light sources in fluorescence microscopy," *Journal of Microscopy-Oxford* **222**, 135-140 (2006).
80. V. Bormuth, J. Howard, and E. Schaffer, "LED illumination for video-enhanced DIC imaging of single microtubules," *Journal of Microscopy* **226**, 1-5 (2007).
81. A. Tsutomu, and M. Hiroaki, "Light emitting diode-based nanosecond ultraviolet light source for fluorescence lifetime measurements," *Rev. Sci. Instrum.* **66**, 5469-5472 (1995).
82. S. Jeffrey, M. C. Gary, R. L. Joseph, and R. Govind, "Single quantum well light emitting diodes demonstrated as excitation sources for nanosecond phase-modulation fluorescence lifetime measurements," *Rev. Sci. Instrum.* **67**, 3795-3798 (1996).
83. C. McGuinness, K. Sagoo, D. McLoskey, and D. Birch, J. S. , "Selective excitation of tryptophan fluorescence decay in proteins using a subnanosecond 295 nm light-emitting diode and time-correlated single-photon counting," *Appl. Phys. Lett.* **86**, 261911 (2005).
84. A. J. Fischer, A. A. Allerman, M. H. Crawford, K. H. A. Bogart, S. R. Lee, R. J. Kaplar, W. W. Chow, S. R. Kurtz, K. W. Fullmer, and J. J. Figiel, "Room-temperature direct current operation of 290 nm light-emitting diodes with milliwatt power levels," *Appl. Phys. Lett.* **84**, 3394-3396 (2004).
85. H. X. Jiang, S. X. Jin, J. Li, J. Shakya, and J. Y. Lin, "III-nitride blue microdisplays," *Appl. Phys. Lett.* **78**, 1303-1305 (2001).
86. S. Strite, and H. Morkoc, "- GaN, AlN, and InN: a review," *Journal of vacuum science and technology -* **10**, 1237 (1992).
87. J. Wu, W. Walukiewicz, K. M. Yu, J. W. Ager Iii, E. E. Haller, H. Lu, W. J. Schaff, Y. Saito, and Y. Nanishi, "Unusual properties of the fundamental band gap of InN," *Appl. Phys. Lett.* **80**, 3967-3969 (2002).
88. E. F. Schubert, "- Light-Emitting Diodes," Cambridge University Press (2006).
89. A. Castiglia, D. Simeonov, H. J. Buehlmann, J. F. Carlin, E. Feltn, J. Dorsaz, R. Butte, and N. Grandjean, "Efficient current injection scheme for nitride vertical cavity surface emitting lasers," *Appl. Phys. Lett.* **90**, 033514-033513 (2007).
90. J. B. Pawley, "- Handbook of biological confocal microscopy," - **31** (2006).
91. O. B. Shchekin, J. E. Epler, T. A. Trottier, T. Margalith, D. A. Steigerwald, M. O. Holcomb, P. S. Martin, and M. R. Krames, "High performance thin-film flip-chip InGaN--GaN light-emitting diodes," *Appl. Phys. Lett.* **89**, 071109 (2006).
92. J. J. Wierer, D. A. Steigerwald, M. R. Krames, J. J. O. Shea, M. J. Ludowise, G. Christenson, Y. C. Shen, C. Lowery, P. S. Martin, S. Subramanya, W. Gotz, N. F. Gardner, R. S. Kern, and S. A. Stockman, "High-power AlGaIn flip-chip light-emitting diodes," *Appl. Phys. Lett.* **78**, 3379-3381 (2001).
93. D. Zakheim, I. Smirnova, I. Roznanskii, S. Gurevich, M. Kulagina, E. Arakcheeva, G. Onushkin, A. Zakheim, E. Vasil'eva, and G. Itkinson, "High-power flip-chip blue light-emitting diodes based on AlGaInN," *Semiconductors* **39**, 851-855 (2005).
94. I. Schnitzer, E. Yablonovitch, C. Caneau, T. J. Gmitter, and A. Scherer, "30% external quantum efficiency from surface textured, thin-film light-emitting diodes," *Appl. Phys. Lett.* **63**, 2174-2176 (1993).
95. M. Khizar, Z. Y. Fan, K. H. Kim, J. Y. Lin, and H. X. Jiang, "Nitride deep-ultraviolet light-emitting diodes with microlens array," *Appl. Phys. Lett.* **86**, 173504 (2005).

96. T. Fujii, Y. Gao, R. Sharma, E. L. Hu, S. P. DenBaars, and S. Nakamura, "Increase in the extraction efficiency of GaN-based light-emitting diodes via surface roughening," *Appl. Phys. Lett.* **84**, 855-857 (2004).
97. R. Windisch, C. Rومان, S. Meinschmidt, P. Kiesel, D. Zipperer, G. H. Dohler, B. Dutta, M. Kuijk, G. Borghs, and P. Heremans, "Impact of texture-enhanced transmission on high-efficiency surface-textured light-emitting diodes," *Appl. Phys. Lett.* **79**, 2315-2317 (2001).
98. M. Shatalov, A. Chitnis, P. Yadav, F. H. Md, J. Khan, V. Adivarahan, H. P. Maruska, W. H. Sun, and M. A. Khan, "Thermal analysis of flip-chip packaged 280 nm nitride-based deep ultraviolet light-emitting diodes," *Appl. Phys. Lett.* **86**, 201109 (2005).
99. H. Peng, "Ultraviolet light-emitting diodes operating in the 340 nm wavelength range and application to time-resolved fluorescence spectroscopy," **85** (2004).
100. S. X. Jin, J. Li, J. Z. Li, J. Y. Lin, and H. X. Jiang, "- GaN microdisk light emitting diodes," *Appl. Phys. Lett.* - **76**, 631-633 (2000).
101. S. X. Jin, J. Li, J. Y. Lin, and H. X. Jiang, "- InGaN/GaN quantum well interconnected microdisk light emitting diodes," *Appl. Phys. Lett.* - **77**, 3236-3238 (2000).
102. I. Ozden, M. Diagne, A. Nurmikko, J. Han, and T. Takeuchi, "A matrix addressable 1024 element blue light emitting InGaN QW diode array," *Physica status solidi. A, Applied research* **188**, 139 (2001).
103. S. J. F. Wu, "Matrix Addressable Micro-Pixel 280 nm Deep UV Light-Emitting Diodes," **45** (2006).
104. H. W. Choi, C. W. Jeon, and M. D. Dawson, "Tapered sidewall dry etching process for GaN and its applications in device fabrication," *Journal of Vacuum Science & Technology B: Microelectronics and Nanometer Structures* **23**, 99-102 (2005).
105. H. W. Choi, C. W. Jeon, and M. D. Dawson, "Fabrication of matrix-addressable micro-LED arrays based on a novel etch technique," *J. Cryst. Growth* **268**, 527-530 (2004).
106. J. M. Benavides, "- Optical characterization of ultrabright LEDs," - **44** (2005).
107. C. F. Shen, S. J. Chang, W. S. Chen, T. K. Ko, C. T. Kuo, and S. C. Shei, "Nitride-Based High-Power Flip-Chip LED With Double-Side Patterned Sapphire Substrate," *Photonics Technology Letters, IEEE* **19**, 780-782 (2007).
108. S. J. Chang, C. S. Chang, Y. K. Su, C. T. Lee, W. S. Chen, C. F. Shen, Y. P. Hsu, S. C. Shei, and H. M. Lo, "Nitride-based flip-chip ITO LEDs," *Advanced Packaging, IEEE* **28**, 273-277 (2005).
109. Improvion, "<http://www.improvion.com/products/vgc>."
110. Zeiss, "www.zeiss.com."
111. Wilson, Neil, and Juskaitis, "Real-time three-dimensional imaging of macroscopic structures," *Journal of Microscopy* **191**, 116-118 (1998).
112. D. Karadaglic, and T. Wilson, "Image formation in structured illumination wide-field fluorescence microscopy," *Micron* **In Press, Corrected Proof**.
113. M. G. L. Gustafsson, "Extended resolution fluorescence microscopy," *Curr. Opin. Struct. Biol.* **9**, 627-634 (1999).
114. S. E. D. Webb, "Development and application of widefield fluorescence lifetime imaging- PhD Thesis," (2003).
115. L. H. Schaefer, D. Schuster, and J. Schaffer, "Structured illumination microscopy: artefact analysis and reduction utilizing a parameter optimization approach," *J. Microsc.-Oxf.* **216**, 165-174 (2004).

116. C. J. Koester, "Scanning mirror microscope with optical sectioning characteristics: applications in ophthalmology," *Appl. Optics* **19**, 1749 (1980).
117. P. A. Benedetti, V. Evangelista, D. Guidarini, and S. Vestri, "Electronic multi-confocal-points microscopy," *SPIE Proceedings* **2412**, 56-62 (1995).
118. M. A. A. Neil, T. Wilson, and R. Juskaitis, "A light efficient optically sectioning microscope," *J. Microsc.-Oxf.* **189**, 114-117 (1998).
119. J. Canny, "A computational approach to edge detection," *IEEE Transactions on Pattern Analysis and Machine Intelligence* **8**, 679-698 (1986).
120. L. Jin, A. C. Millard, J. P. Wuskell, H. A. Clark, and L. M. Loew, "Cholesterol-Enriched Lipid Domains Can Be Visualized by di-4-ANEPPDHQ with Linear and Nonlinear Optics," *Biophys. J.* **89**, L04-06 (2005).
121. L. Jin, A. C. Millard, J. P. Wuskell, X. Dong, D. Wu, H. A. Clark, and L. M. Loew, "Characterization and Application of a New Optical Probe for Membrane Lipid Domains," *Biophys. J.* **90**, 2563-2575 (2006).
122. D. M. Owen, P. M. P. Lanigan, C. Dunsby, I. Munro, D. Grant, M. A. A. Neil, P. M. W. French, and A. I. Magee, "Fluorescence Lifetime Imaging Provides Enhanced Contrast when Imaging the Phase-Sensitive Dye di-4-ANEPPDHQ in Model Membranes and Live Cells," *Biophys. J.* **90**, L80-82 (2006).
123. A. L. Hodgkin, and A. F. Huxley, "A quantitative description of membrane current and its application to conduction and excitation in nerve," *J Physiol* **117**, 500-544 (1952).
124. J. J. H. Kaplan, B. B. Forbush, and J. J. F. Hoffman, "- Rapid photolytic release of adenosine 5'-triphosphate from a protected analogue: utilization by the Na:K pump of human red blood cell ghosts," *Biochemistry* **17**, 35-45 (1978).
125. S. Shoham, "- Rapid neurotransmitter uncaging in spatially defined patterns," - **2** (2005).
126. M. Banghart, K. Borges, E. Isacoff, D. Trauner, and R. H. Kramer, "Light-activated ion channels for remote control of neuronal firing," *Nat Neurosci* **7**, 1381-1386 (2004).
127. R. G. Foster, "Neurobiology: Bright blue times," *Nature* **433**, 698-699 (2005).
128. F. Zhang, L.-P. Wang, M. Brauner, J. F. Liewald, K. Kay, N. Watzke, P. G. Wood, E. Bamberg, G. Nagel, A. Gottschalk, and K. Deisseroth, "Multimodal fast optical interrogation of neural circuitry," *Nature* **446**, 633-639 (2007).
129. X. Han, and E. S. Boyden, "Multiple-Color Optical Activation, Silencing, and Desynchronization of Neural Activity, with Single-Spike Temporal Resolution," *PLoS ONE* **2**, e299 (2007).
130. E. S. Boyden, F. Zhang, E. Bamberg, G. Nagel, and K. Deisseroth, "Millisecond-timescale, genetically targeted optical control of neural activity," *Nat Neurosci* **8**, 1263-1268 (2005).
131. E. Korkotian, D. Oron, Y. Silberberg, and M. Segal, "Confocal microscopic imaging of fast UV-laser photolysis of caged compounds," *J. Neurosci. Methods* **133**, 153-159 (2004).
132. T. T. Ishizuka, M. M. Kakuda, R. R. Araki, and H. H. Yawo, "- Kinetic evaluation of photosensitivity in genetically engineered neurons expressing green algae light-gated channels," - **54**, - 94 (2006).
133. G. C. R. Ellis-Davies, "Caged compounds: photorelease technology for control of cellular chemistry and physiology," *Nat Meth* **4**, 619-628 (2007).
134. G. C. R. Ellis-Davies, and J. H. Kaplan, "Nitrophenyl-EGTA, A Photolabile Chelator that Selectively Binds Ca²⁺ with High Affinity and Releases it Rapidly Upon Photolysis," *Proc. Natl. Acad. Sci. U. S. A.* **91**, 187-191 (1994).

135. R. Wieboldt, "Photolabile precursors of glutamate: synthesis, photochemical properties, and activation of glutamate receptors on a microsecond time scale," *Proc. Natl. Acad. Sci. U. S. A.* **91**, 8752-8756 (1994).
136. H. Kasai, "Comparative biology of Ca²⁺-dependent exocytosis: implications of kinetic diversity for secretory function," *Trends in Neurosciences* **22**, 88-93 (1999).
137. E. M. Callaway, and L. C. Katz, "Photostimulation Using Caged Glutamate Reveals Functional Circuitry in Living Brain Slices," *PNAS* **90**, 7661-7665 (1993).
138. E. B. Brown, J. B. Shear, S. R. Adams, R. Y. Tsien, and W. W. Webb, "Photolysis of Caged Calcium in Femtoliter Volumes Using Two-Photon Excitation," *Biophysical Journal* **76**, 489-499 (1999).
139. J. L. Puchalla, E. Schneidman, R. A. Harris, and M. J. Berry, "Redundancy in the Population Code of the Retina," *Neuron* **46**, 493-504 (2005).
140. A. Samadani, J. Mettetal, and A. van Oudenaarden, "Cellular asymmetry and individuality in directional sensing," *PNAS* **103**, 11549-11554 (2006).
141. Y. Y. Bernardinelli, C. C. Haerberli, and J. Y. J.-Y. Chatton, "- Flash photolysis using a light emitting diode: an efficient, compact, and affordable solution," - **37**, - 72 (2005).
142. Y. Yoshimura, J. L. M. Dantzker, and E. M. Callaway, "Excitatory cortical neurons form fine-scale functional networks," *Nature* **433**, 868-873 (2005).
143. B. P. Bean, "The action potential in mammalian central neurons," *Nat Rev Neurosci* **8**, 451-465 (2007).
144. S. Panda, S. K. Nayak, B. Campo, J. R. Walker, J. B. Hogenesch, and T. Jegla, "Illumination of the Melanopsin Signaling Pathway," *Science* **307**, 600-604 (2005).
145. X. Qiu, T. Kumbalasiri, S. M. Carlson, K. Y. Wong, V. Krishna, I. Provencio, and D. M. Berson, "Induction of photosensitivity by heterologous expression of melanopsin," *Nature* **433**, 745-749 (2005).
146. M. S. Freedman, R. J. Lucas, B. Soni, M. von Schantz, Mu, ntilde, M. oz, Z. David-Gray, euml, and R. Foster, "Regulation of Mammalian Circadian Behavior by Non-rod, Non-cone, Ocular Photoreceptors," *Science* **284**, 502-504 (1999).
147. R. J. Lucas, M. S. Freedman, Mu, ntilde, M. oz, F. Garcia, aacute, J. ndez, eacute, M., and R. G. Foster, "Regulation of the Mammalian Pineal by Non-rod, Non-cone, Ocular Photoreceptors," *Science* **284**, 505-507 (1999).
148. R. J. Lucas, R. H. Douglas, and R. G. Foster, "Characterization of an ocular photopigment capable of driving pupillary constriction in mice," *Nat Neurosci* **4**, 621-626 (2001).
149. Z. Melyan, E. E. Tarttelin, J. Bellingham, R. J. Lucas, and M. W. Hankins, "Addition of human melanopsin renders mammalian cells photoresponsive," *Nature* **433**, 741-745 (2005).
150. G. Nagel, T. Szellas, W. Huhn, S. Kateriya, N. Adeishvili, P. Berthold, D. Ollig, P. Hegemann, and E. Bamberg, "Channelrhodopsin-2, a directly light-gated cation-selective membrane channel," *Proceedings of the National Academy of Sciences* **100**, 13940-13945 (2003).
151. K. Nikolic, P. Degenaar, and C. Toumazou, "- Modeling and Engineering aspects of ChannelRhodopsin2 System for Neural Photostimulation," -, - 1629 (2006).
152. A. Bi, J. Cui, Y.-P. Ma, E. Olshevskaya, M. Pu, A. M. Dizhoor, and Z.-H. Pan, "Ectopic Expression of a Microbial-Type Rhodopsin Restores Visual Responses in Mice with Photoreceptor Degeneration," *Neuron* **50**, 23-33 (2006).
153. B. Sakmann, and E. Neher, *Single-channel recording* (Plenum Press, New York, 1995).

154. J. G. Nicholls, A. R. Martin, B. G. Wallace, and P. A. Fuchs, *From neuron to brain* (Sinauer Associates, Sunderland, USA, 2000).
155. K. N. Hartman, S. K. Pal, J. Burrone, and V. N. Murthy, "Activity-dependent regulation of inhibitory synaptic transmission in hippocampal neurons," *Nat Neurosci* **9**, 642-649 (2006).
156. C. C. A. Thomas, P. P. A. Springer, G. G. E. Loeb, Y. Y. Berwald-Netter, and L. L. M. Okun, "- A miniature microelectrode array to monitor the bioelectric activity of cultured cells," - **74**, - 6 (1972).
157. F. Hofmann, and H. Bading, "Long term recordings with microelectrode arrays: Studies of transcription-dependent neuronal plasticity and axonal regeneration," *Journal of Physiology-Paris* **99**, 125-132 (2006).
158. F. Lante, M. C. De Jesus Ferreira, J. Guiramand, M. Recasens, and M. Vignes, "Low-frequency stimulation induces a new form of LTP, metabotropic glutamate receptor- and PKA-dependent, in the CA1 area of the rat hippocampus," *Hippocampus* **16**, 345-360 (2006).
159. J. Cang, R. C. Renteria, M. Kaneko, X. Liu, D. R. Copenhagen, and M. P. Stryker, "Development of Precise Maps in Visual Cortex Requires Patterned Spontaneous Activity in the Retina," *Neuron* **48**, 797-809 (2005).
160. S. J. Aton, C. S. Colwell, A. J. Harmar, J. Waschek, and E. D. Herzog, "Vasoactive intestinal polypeptide mediates circadian rhythmicity and synchrony in mammalian clock neurons," *Nat Neurosci* **8**, 476-483 (2005).
161. G.-q. Bi, and M.-m. Poo, "Synaptic Modifications in Cultured Hippocampal Neurons: Dependence on Spike Timing, Synaptic Strength, and Postsynaptic Cell Type," *Journal of Neuroscience* **18**, 10464-10472 (1998).
162. G. Q. Bi, and M. M. Poo, "Distributed synaptic modification in neural networks induced by patterned stimulation," *Nature* **401**, 792 (1999).
163. P. E. Latham, B. J. Richmond, P. G. Nelson, and S. Nirenberg, "Intrinsic Dynamics in Neuronal Networks. I. Theory," *Journal of Neurophysiology* **83**, 808-827 (2000).
164. T. T. H. Müller, U. U. Misgeld, and D. D. Swandulla, "Ionic currents in cultured rat hypothalamic neurones," *The Journal of physiology* **450**, 341-362 (1992).
165. T. DeMarse, D. Wagenaar, A. Blau, and S. Potter, "The Neurally Controlled Animat: Biological Brains Acting with Simulated Bodies," *Autonomous Robots* **11**, 305-310 (2001).
166. S. Potter, D. Wagenaar, and T. DeMarse, "Closing the Loop: Stimulation Feedback Systems for Embodied MEA Cultures," in *Advances in Network Electrophysiology*(2006), pp. 215-242.
167. E. Zrenner, "Will Retinal Implants Restore Vision?," *Science* **295**, 1022-1025 (2002).
168. A. Y. Chow, V. Y. Chow, K. H. Packo, J. S. Pollack, G. A. Peyman, and R. Schuchard, "The Artificial Silicon Retina Microchip for the Treatment of Vision Loss From Retinitis Pigmentosa," *Archives of Ophthalmology* **122**, 460-469 (2004).
169. J. D. Weiland, W. Liu, and M. S. Humayun, "Retinal prosthesis," *Annual Review of Biomedical Engineering* **7**, 361-401 (2005).
170. J. Christ, and S. T. Matthew, "Healing blindness: an experimental approach," *Journal of Biblical Therapy* **1**, 20-30 (323).
171. H. Kolb, E. Fernandez, and R. Nelson, "www.webvision.med.utah.edu."
172. C. Veraart, F. Duret, M. Brelen, M. Oozeer, and J. Delbeke, "Vision rehabilitation in the case of blindness," *Expert Review of Medical Devices* **1**, 139-153 (2004).

173. G. The Eye Diseases Prevalence Research, "Prevalence of Age-Related Macular Degeneration in the United States," *Archives of Ophthalmology* **122**, 564-572 (2004).
174. M. M. Kalloniatis, and E. L. E. L. Fletcher, "Retinitis pigmentosa: understanding the clinical presentation, mechanisms and treatment options," *Clinical & experimental optometry* **87**, 65-80 (2004).
175. A. Y. Chow, M. T. Pardue, V. Y. Chow, G. A. Peyman, C. Liang, J. I. Perlman, and N. S. Peachey, "Implantation of silicon chip microphotodiode arrays into the cat subretinal space," *Neural Systems and Rehabilitation Engineering, IEEE Transactions on* [see also *IEEE Trans. on Rehabilitation Engineering*] **9**, 86-95 (2001).
176. M. S. Humayun, E. de Juan Jr, J. D. Weiland, G. Dagnelie, S. Katona, R. Greenberg, and S. Suzuki, "Pattern electrical stimulation of the human retina," *Vision Research* **39**, 2569-2576 (1999).
177. P. Degenaar, M. W. Hankins, E. Drakakis, C. Toumazou, K. Nikolic, C. Kennard, and Y. Huang, "Retinal prosthetics devices," (UK, 2007).
178. B. L. B. L. Davidson, and X. O. X. O. Breakefield, "Viral vectors for gene delivery to the nervous system," *Nature reviews. Neuroscience* **4**, 353-364 (2003).
179. C. Moller, and A. Travis, "Flat Panel Time Multiplexed Autostereoscopic Display Using an Optical Wedge® Waveguide," *Proc. 11 thInternational Display Workshop* (2004).

Appendix 1: Analytical approximation of the diffraction optical transfer function (OTF)

The response of a defocused aberration-free incoherent optical imaging system to line-frequencies in the object was studied analytically and derived by Hopkins in case of low numerical aperture focusing. Hopkins described the OTF as a series of Bessel functions:

$$D(s, u) = \frac{4}{\pi u s} \cos\left(\frac{1}{2} u s^2\right) \left\{ \beta J_1(us) + \sum_{n=1}^{\infty} (-1)^{n+1} \frac{\sin(2n\beta)}{2n} [J_{2n-1}(us) - J_{2n+1}(us)] \right\} \\ - \frac{4}{\pi u s} \sin\left(\frac{1}{2} u s^2\right) \left\{ \sum_{n=0}^{\infty} (-1)^n \frac{\sin((2n+1)\beta)}{2n+1} [J_{2n}(us) - J_{2(n+1)}(us)] \right\} \quad (9.1)$$

where s is the spatial frequency of the line pattern and u is the normalised defocus of the imaging system. By modifying the geometrical OTF and comparing it with the exact OTF, Stokseth empirically derived an analytical approximation that is much easier to compute.

$$T(s, u) = \begin{cases} g(|s|) \frac{2J_1\left[\frac{us(1-|s|/2)}{1-|s|/2}\right]}{us(1-|s|/2)} & \text{if } 0 < |s| < 2 \\ 0 & \text{otherwise} \end{cases} \quad (9.2)$$

$$g(s) = 1 - 0.69s + 0.0076s^2 + 0.043s^3$$

This OTF was shown to be very accurate to intermediate and large defocus, for which the exact solution is the most time consuming to evaluate. Fig. 8.1 shows that both the exact function and its approximation overlap when a large amount of defocus is present. Conversely, a difference up to 10% can be noticed between the exact OTF and the approximation for a small amount of defocus.

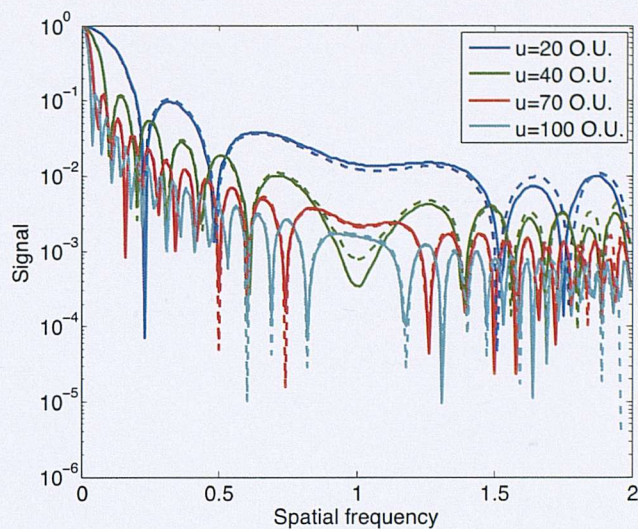


Fig. 8.1. Hopkins (plain) and Stokseth (dashed) optical transfer functions at large defocus

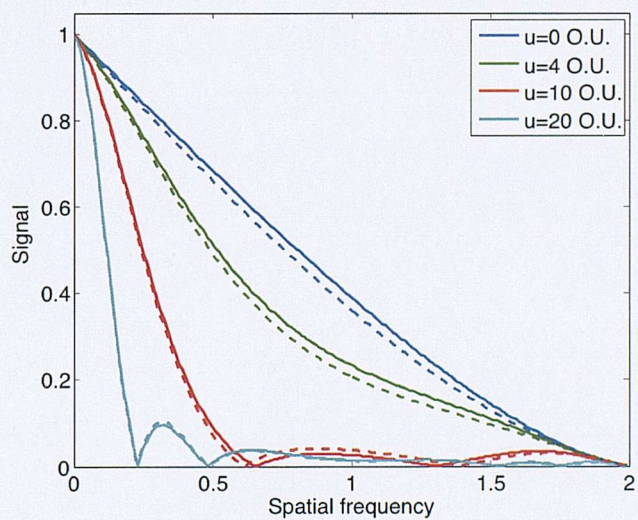


Fig. 8.2. Hopkins (plain) and Stokseth (dashed) OTFs in presence of small defocus

Publications and presentations

Journal publications

V. Poher, H. X. Zhang, G. T. Kennedy, C. Griffin, S. Oddos, E. Gu, D. S. Elson, J. M. Girkin, P. M. W. French, M. D. Dawson and M. A. A. Neil (2007) "Optical sectioning microscopes with no moving parts using a micro-stripe array light emitting diode", *Optics Express*: 15 11196-11206

V. Poher, G. T. Kennedy, D. S. Elson, P. M. W. French, M. A. A. Neil, H. X. Zhang, E. Gu, Z. Gong, C. Griffin, J. M. Girkin and M. D. Dawson (2007) "Microscopy using micropixelated light emitting diodes", *Proceedings of SPIE*: **6443** 644337

V. Poher, N. Grossman, G. T. Kennedy, K. Nikolic, H. X. Zhang, Z. Gong, E. M. Drakakis, E. Gu, M. D. Dawson, P. M. W. French, P. Degenaar and M. A. A. Neil (2008) "Micro-LED arrays: a tool for two-dimensional neuron stimulation", *Journal of Physics D*: **41** 094014

V. Poher, G. T. Kennedy, H. B. Manning, D. M. Owen, H. X. Zhang, E. Gu, M. D. Dawson, P. M. W. French and M. A. A. Neil (2008) "Improved sectioning in a slit scanning confocal microscope", *Optics letters*: **Accepted**, In press

Z. Gong, H. X. Zhang, E. Gu, C. Griffin, M. D. Dawson, V. Poher, G. T. Kennedy, P. M. W. French and M. A. A. Neil, (2007) "Matrix-Addressable Micropixelated InGaN Light-Emitting Diodes With Uniform Emission and Increased Light Output" *Electron Devices, IEEE*: **54** 2650-2658

Z. Gong, E. Gu, S. R. Jin, D. Massoubre, B. Guilhabert, H. X. Zhang, M. D. Dawson, V. Poher, G. T. Kennedy, P. M. W. French and M. A. A. Neil (2008) "Efficient flip-chip InGaN micro-pixelated light-emitting diode arrays: promising candidates for micro-displays and colour conversion" (2008), *Journal of Physics D*: **41** 094002

G. T. Kennedy, D. S. Elson, J. D. Hares, I. Munro, V. Poher, P. M. W. French and M. A. A. Neil (2008) "Fluorescence lifetime imaging using light emitting diodes", *Journal of Physics D*: **41** 094012

Conference presentations

V. Poher, D. S. Elson, P. M. P. Lanigan, C. Dunsby, P. M. W. French and M. A. A. Neil, "Shot noise in widefield structured illumination microscopy: an intrinsic limitation for quantitative measurements? ", Poster presentation, *Focus On Microscopy*, Jena, Germany (2005)

V. Poher, G. T. Kennedy, D. S. Elson, P. M. W. French, M. A. A. Neil, H.Z. Zhang, E. Gu, Z. Gong, C. Griffin, J. M. Girkin, and M. D. Dawson, "Structured illumination microscopy using light emitting diodes", *Photon06*, *Institute of Physics*: Manchester, UK (2006)

G. T. Kennedy, D. S. Elson, I. Munro, V. Poher, P. M. W. French and M. A. A. Neil, "Fluorescence Lifetime Imaging Using Light Emitting Diodes" *Photon06*, *Institute of Physics*: Manchester, UK (2006)

V. Poher, G. T. Kennedy, D. S. Elson, P. W. French, M. A. A. Neil, "LEDs trip the light fantastic", Poster presentation, *House of Commons*, UK (2006)

V. Poher, G. T. Kennedy, D. S. Elson, P. M. W. French, M. A. A. Neil, H. X. Zhang, E. Gu, Z. Gong, C. Griffin, J. M. Girkin and M. D. Dawson, "Microscopy using micropixelated light emitting diodes", *Photonics West: BIOS*, San Jose, USA (2007)

V. Poher, G. T. Kennedy, P. M. W. French, M. A. A. Neil, E. Gu, and M. D. Dawson, "Microscopy using micro-pixelated light emitting diodes", *UK Nitride Consortium*, Oxford, UK (2007)

V. Poher, G. T. Kennedy, D. S. Elson, P. M. W. French, M. A. A. Neil, H. X. Zhang, E. Gu, Z. Gong, C. Griffin, J. M. Girkin and M. D. Dawson, "LED light sources for fluorescence microscopy", *Focus On Microscopy*, Valencia, Spain (2007)

V. Poher, G. T. Kennedy, D. S. Elson, P. M. W. French, M. A. A. Neil, H. X. Zhang, E. Gu, Z. Gong, C. Griffin, J. M. Girkin and M. D. Dawson, "LED light sources for fluorescence microscopy", *European Conference on Biomedical Optics*, Munich, Germany (2007)

V. Poher, G. T. Kennedy, D. S. Elson, P. M. W. French, M. A. A. Neil, H. X. Zhang, E. Gu, Z. Gong, C. Griffin, J. M. Girkin and M. D. Dawson, "Micro-structured Light emitting Diode arrays: Applications in microscopy", *Optical Micro Systems 2007*, Capri, Italy (2007)

V. Poher, G. T. Kennedy, H. B. Manning, D. M. Owen, P. M. W. French and M. A. A. Neil, "Method of improving the axial resolution and image contrast in a slit scanning confocal microscope" *Focus On Microscopy*, Osaka, Japan (2008)