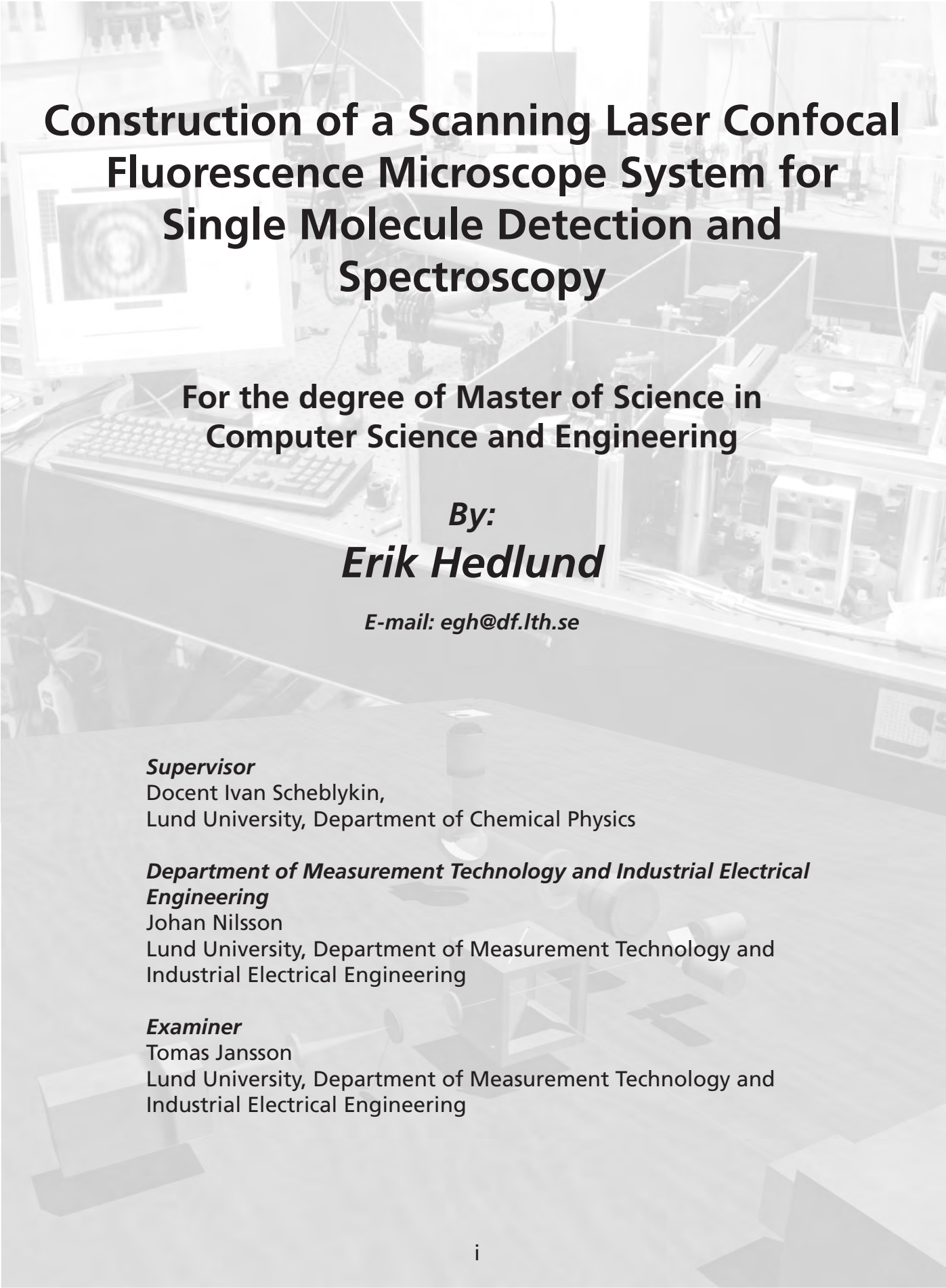




Construction of a Scanning Laser Confocal Fluorescence Microscope System for Single Molecule Detection and Spectroscopy

For the degree of Master of Science in
Computer Science and Engineering

By:
Erik Hedlund

E-mail: egh@df.lth.se

Supervisor

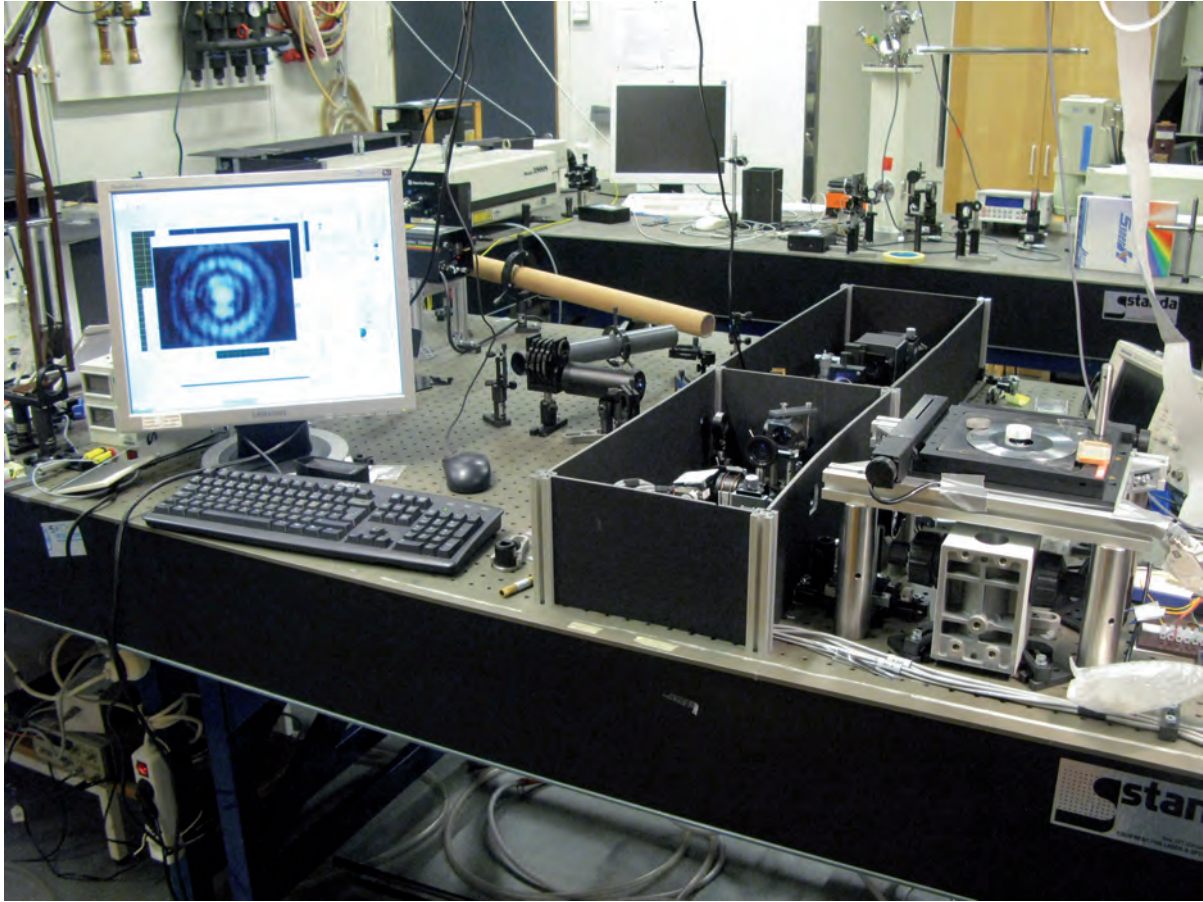
Docent Ivan Scheblykin,
Lund University, Department of Chemical Physics

***Department of Measurement Technology and Industrial Electrical
Engineering***

Johan Nilsson
Lund University, Department of Measurement Technology and
Industrial Electrical Engineering

Examiner

Tomas Jansson
Lund University, Department of Measurement Technology and
Industrial Electrical Engineering



Abstract

The Single Molecule Spectroscopy Group at the department of Chemical Physics in the Chemical Centre of Lund University conducts research involving spectroscopy on single molecules of organic dyes.

Fluorescence microscopy using a wide-field scheme has been used and such a scheme has many advantages such as full image capture at once when using a high sensitivity CCD or CMOS camera. Using different techniques it may be possible to conduct more sensitive analysis on single points in such an image using a photo multiplier tube (PMT) or an avalanche photo diode (APD).

It is desirable to have the possibility to analyse the spectral properties of single molecules of organic dyes, a sensitivity not attained by the wide-field strategies currently used at the SMS group.

A confocal strategy has the advantage of a very shallow depth of field. The use of a pinhole for selecting a single plane of the sample, ideally diffraction limited, minimises the problem pertaining to stray light from the surroundings and effects of fluorescing sample carriers (glass plates). Thus the strategy can potentially greatly improve the signal to noise ratio in single molecule spectroscopy applications.

The power intensity of the laser light normally used for excitation in fluorescence microscopy tend to be relatively high and problems with photo bleaching arise. Especially when analysing single points on the sample there is really no reason to illuminate the whole sample, risking bleaching of the entire sample, when only the analysed molecule needs illumination.

To combine the benefits of a sensitive detector such as a PMT or APD with lowest possible total light dose and intermittent exposure of the sample a scanning confocal setup has been suggested and proven effective.

A further advantage of a confocal strategy is the possibility to use high power pulsed lasers and two-photon processes to analyse samples. The use of two-photon processes would be, if not impossible, extremely impractical (and also dangerous if used in an open setup) in a wide-field setup as the light would be highly divergent and the optical power thus needs to be much higher to obtain the needed power density at the sample.

This thesis covers the construction and optimisation of a scanning laser confocal fluorescence microscope system as well as a redesign and optimisation of a LabVIEW based control scheme presented by Giang Vu in his Masters thesis *Software Control of a Confocal Microscope* [1].

Table of Contents

Abstract	iii
1. Introduction	1
1.1 Background	1
1.2 Confocal Microscopy for Single Molecule Spectroscopy	1
1.3 Earlier Work	2
1.4 Similar Constructions	3
1.5 Constructing the Microscope	4
2. Theory	5
2.1 Confocal Microscopes	5
2.2 Resolution	6
2.3 Sensitivity	12
2.4 Angular and Spatial Resolution	12
2.5 Speckle	14
2.6 The Dose Concept	14
2.7 Simplifications	16
2.8 Spectroscopic Considerations	17
3. Implementation – Hardware	19
3.1 The Microscope Blocks	20
3.2 Aligning	22
3.3 Focusing	23
4. Implementation – Software	27
4.1 Basic Issues	27
4.2 Needed Performance	28
4.3 Communication Protocols	29
4.3.1 <i>Interface Properties</i>	30

4.4	Structure	31
4.4.1	<i>Block 1 - Parameter Entry</i>	36
4.4.2	<i>Block 2 - Scanning</i>	36
4.4.3	<i>Block 3 - Image Analysis, Aiming and Single Point Analysis</i>	
	<i>(Live capture of single points)</i>	38
4.5	File Formats	39
4.5.1	<i>Image Files</i>	39
4.5.2	<i>Single Point Files</i>	40
4.5.3	<i>The Pseudo EXIF File</i>	40
5.	Results and Conclusions	41
5.1	Sensitivity	41
5.3	Software	41
5.4	Images	42
6.	Further Development	47
6.1	Hardware	47
6.2	Software	48
Appendix		49
A.1	Microscope Imaging	49
A.1.1	<i>Contrast</i>	49
A.1.2	<i>The Point Spread Function</i>	49
A.2	Absorption Cross Section	50
B.	Hardware List	51
Bibliography		53

*“If time were to stand still
the World would be awfully dark...”*



1. Introduction

1.1 Background

At the Department for Chemical Physics at Lund University research has been conducted for some time on various polymers with e.g. the potential of acting as semiconductors under some circumstances. For various spectroscopic analyses a wide-field microscope system has been used to image the fluorescence of singular molecules (polymer chains) in thin films of organic polymers. The samples are low concentration with a distribution of only a few molecules per μm^2 . A wish to be able to lower the background interference and have the opportunity to use two-photon processes led to the idea to use a scanning confocal scheme. This would make it possible to use lower power in the excitation beam and minimise the exposure time as every single point would only need to be exposed to light when being scanned for the overview image acquisition. Single point analysis could then be used with one- or two-photon processes as needed. Also, organic polymers are sensitive to photo bleaching at relatively low powers, so a lower light dose and intermittent illumination could possibly prolong the life of a sample.

1.2 Confocal Microscopy for Single Molecule Spectroscopy

The project was conceived as a diploma project by Ivan Scheblykin. The whole concept revolves around a single sided laser excited scanning confocal fluorescence microscope. It was envisioned that mostly standard optics would be needed. The final setup uses several standard parts from Olympus microscopes for the visible regime. In figure 1.2 the basic design of the system is outlined.

The confocal scheme is perfectly suited to use an avalanche photo diode (APD) as a sensor as no XY-resolution (as on e.g. a CCD chip) is needed. An APD is not as sensitive as an intensified CCD chip (quantum efficiency for the APD is ca 40% whereas a CCD can be as high as 90%) but this concern is lessened by the fact that the confocal scheme have very shallow depth of field and allows for time resolved measurements. The fluorescence from any particle is emitted isotropically, but the only interesting part of the emitted fluorescence is the beam that exactly retraces the excitation light and the idea is to focus as much of this (preferably all available) light onto the APD's active spot.

Even though the light intensity and *dose* per area unit is equal to the wide-field scheme when imaging, the sensitivity is greatly enhanced due to the very shallow depth of field in the confocal scheme, which is determined by the size of the pinhole used and the wavelength. This is due to the fact that unwanted fluorescence from out of focus points is efficiently suppressed. In practice a diffraction limited volume can be selected and analysed with little interference from surrounding chromophores. Although the setup described in this thesis actually has an imaging dose of light that is roughly equal to the dose delivered in the wide-field counterpart the average total illumination dose is actually somewhat lowered as no wide-field illumination is needed when focusing and when analysing specific points on the sample.

On a further note it shall be pointed out that in the scheme devised and de-

scribed in this paper no wavelength separation of the fluorescence apart from the dichroic mirror sectioning the detector line from the microscope and scanning apparatus is done. All fluorescence of sufficiently long wavelengths to pass through the dichroic mirror and the interference filters following it (the exact functions are further discussed in section 3 covering the construction of the setup) are detected. Simple spectroscopic analysis can be performed by choosing appropriate filters and further improvements of the spectroscopic resolution is discussed later in section 6, Further Development.

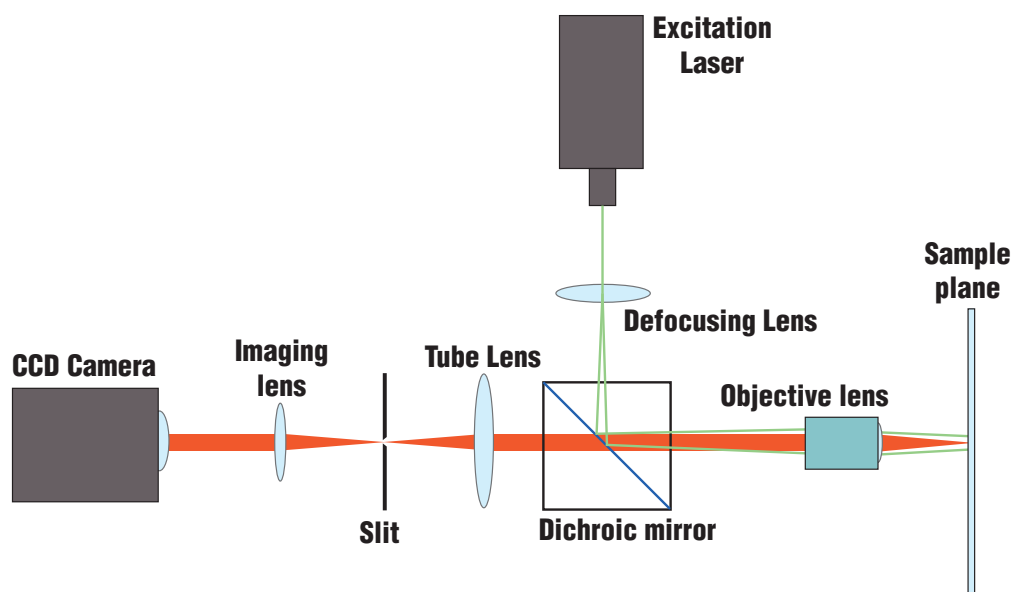


Figure 1.1 The basic widefield strategy used at Chemical Physics. Note the whole sample is illuminated throughout the experiment (beam angles grossly exaggerated). Red beams show the imaging of a point.

1.3 Earlier Work

The project at hand was aimed at trying a new approach to the research based on analysis of single molecules of polymers and dyes at the department of Chemical Physics at Lund University. MEH-PPV chains has been studied extensively by amongst others Ivan Scheblykin, Oleg Mirzov and Daniel Thomson. The project was initialised by Docent Ivan Scheblykin in late 2007. A prototype for the scanning software was devised by Giang Vu, in his masters thesis in electrical engineering [1].

Mr. Vu's software demonstrated the principles needed for the microscope to function, yet it was unfortunately not very user friendly as it was highly modularised with several different user interfaces and no interconnecting software. As it was constructed, the software seemed to give correct results but it soon proved not to be particularly useful when collecting live data. Mr. Vu's thesis addressed several of the characteristics of the used hardware and these experiences proved very valuable when redesigning the controlling software. Further it was a great advantage to have a partially working system when beginning to build the actual microscope setup.

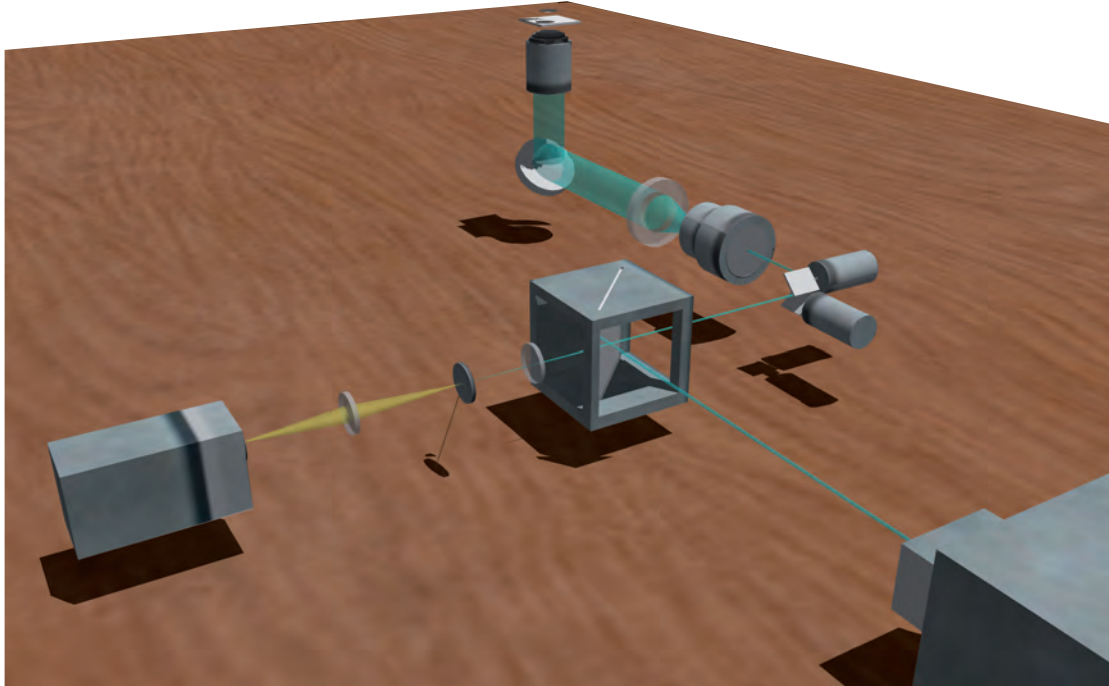


Figure 1.2 Depicting the basic layout of the microscope setup. In the lower right corner is the light source, to the left the APD with optics and pinhole, at the centre the dichroic mirror, centre right: scanning mirrors, top: infinity corrected microscope

1.4 Similar Constructions

The scanning confocal scheme has been around for some time and has become quite popular in biological and biomedical applications due to its high spatial resolution and shallow depth of field, even allowing for sequential acquiring of three dimensional images. Long wavelengths have proven particularly good in biological applications since the scattering of shorter wavelengths tend to be much higher [8]. However in a spectroscopy application the wavelength can be selected arbitrarily from the IR region to the UV range (provided that the sample is planar, e.g. smeared or spincoated onto a glass plate).

Several scanning strategies are viable and have been used to varying extent in real world applications. The most straightforward methods use either a direct manipulation of the sample and a fixed optical system where the sample is moved using e.g. a piezo-crystal actuated XY(Z)-stage, the Z-axis being optional, providing focusing and possibly 3D-scanning provisions.

The other approach is to use a stationary sample and provide some means of manipulating the position of the illuminated spot in the (stationary) sample plane. The former strategy is a simple yet effective setup with few optical elements but the mechanics of it is unsuitable for the purpose intended for the microscope constructed.

In the stationary sample scheme the illumination spot movements are performed by altering the angles of the excitation light beam sent through the microscope. The angles are generally controlled by moveable mirrors. This precision part can either use an

oscillative method, where the mirrors angles follow a precise and predictable trajectory between two endpoints, the method is favoured in constructions for video rate imaging. It can use rotating mirrors in combinations [2][3]. Or it can, like in the system described in this report (described in depth in section 3), use individually settable mirror angles, controlled with high precision galvanometers. This might introduce a somewhat larger overhead as compared to the oscillating processes, possibly not allowing for video rate imaging, but gives a larger freedom for the constructor and end user to select points of interest.

1.5 Constructing the Microscope

The main purpose of the project covered by this masters project was to practically construct a laser scanning confocal microscope suitable for the application at hand and make it usable in a controlled and stationary laboratory environment. The usability criterion is primarily fulfilled due to the introduction of easy-to-use software adhering to the principle *One application, one interface*. Much effort was put into consolidating several software stubs into a single intuitive user interface.

The physical construction of the microscope took some time since everything was made basically from scratch. A basic layout (shown in figure 1.2 and more in detail in figure 3.1) was agreed upon and after that problems and difficulties were addressed as they occurred. Considerable time was spent on laying out the optical axes and calibrating them. The full recount is presented in section 3. One point that tended to be problematic when setting up the optical system was the fact that the whole idea of the system is that a full image never is supposed to be acquired. It is supposed to be sequentially scanned, point by point and it is hard to use imaging as an aid when aligning some parts due to small mirrors and long axes. Also focusing is a nontrivial task and some different methods were tried out.

Software construction initially consisted of adapting mr. Vu's solution for a real world setting. Mr. Vu constructed a system where scan sequences had to be preprogrammed into the mirror controller which then ran in a standalone manner. A few different methods for starting a preprogrammed scan sequence was tested. As none of them was fully satisfactory, a fully integrated LabVIEW controlled system was devised. Some parts of the present software are still extensively based on Vu's work, such as the way the imaging matrices works but the whole mirror control system was rewritten and several settings and indicators were added to provide an easily understandable user interface.

Most of the LabVIEW Virtual Instruments (VIs) provided by GSI Lumonics where sparsely documented so it took some investigative work (to the edge of reverse engineering) to find the functions and proper use of many of them. Initially this discouraged the use of a directly LabVIEW controlled scheme such as the one finally constructed. But as the requirements on the project software grew the preprogrammed method proved to be very inflexible and problematic to use. Some trial and error was also necessary before the present configuration saw the light of day. The current software controls every aspect of the image acquisition except laser power output from within the LabVIEW program.

2. Theory

In this project the objective was to construct a confocal microscope setup being able to study fluorescence from singular particles much smaller than the wavelength at which they fluoresce. This means that the light emitting structures will behave like point sources and provided that samples are spread out sufficiently sparse single molecules can be independently analysed.

A microscope is used to image the point sources and the Rayleigh criterion applies as the wavefronts sent out from two adjacent molecules is imaged. Thus the spacing between the particles will need to be more than 1.22 times the wavelength of the fluorescence in a traditional microscope. The usage of a confocal strategy actually gives a slightly better resolution due to it imaging point by point and not the whole plane at once [2] See further elaboration of this in section 2.2 - Resolution.

An important detail to remember is that all images of single molecules are in fact images of the Airy-pattern created by the molecule acting as a point source, as described in this section. Of course the structure of the molecule itself, being much smaller than the imaging wavelength, cannot be imaged using this technique.

2.1 Confocal Microscopes

The basic principles of an optical scanning confocal microscope are the same as for any optical microscope. Two basic principles are prevalent, the *finite corrected* and the *infinity corrected* schemes.

In the older principle, the *finite corrected* system an *Objective Lens* or lens system directly creates an intermediate image of the sample plane, which is viewed through a second lens system, the *Imaging lens*, also known as *Eyepiece* or *Ocular*. This is a simple scheme with few optical elements, yet it has some disadvantages. E.g. Magnification is not constant at different focal planes and it is hard to introduce secondary optical elements in the beampath.

These disadvantages are addressed by the *infinity corrected* scheme. The idea is to introduce a third major optical component in the beampath, the *Tube Lens*. The tube lens is placed after the objective lens which no longer produces an image of the sample plane but a parallel beam, i.e. it can be considered to be focused to infinity, hence the name of the principle. The tube lens then creates the intermediate image which just as before is viewed through the imaging lens. This scheme is shown in figure 2.1 and 3.1. The parallel beam between the objective and tube lenses caters for a space where the beam behaves consistently, in this part non-imaging optical components such as filters and polarisers can be inserted. In the case of the microscope constructed in this project, only a planar mirror, changing the direction of the beampath is inserted in this space.

The distance between the objective and tube lenses is called the *Tube Length* and is in commercially available solutions generally close to the focal length of the tube lens (a very common focal length is 160mm), though minor discrepancies are of little or no concern. The advantage of placing the tube lens at its focal length from the objective

lens becomes obvious when operating in a confocal scheme due to the fact the angular control of the illuminated spot. If the tube lense's second focus coincides with the ocular's first focal point and finally the scanning mirrors are placed at the ocular's second focal plane, the result is that the small and precise angular movements at the scanning mirrors translates to a much smaller angle at the objective lens and corresponding lateral movement at the sample plane with certain advantages at the intermediate optical components. The laser used as excitation light can be considered to emanate from a point source and being a approximately parallel gaussian beam. The principle is illustrated in figure 2.1, the green outlines represent three different angles in one plane, at $\theta^{x,y} = 0^\circ$ and

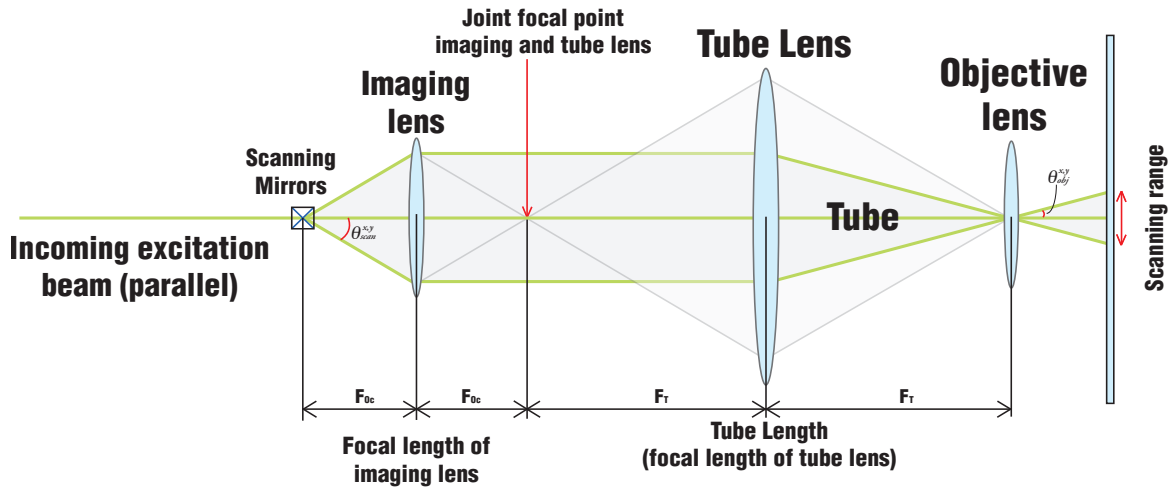


Figure 2.1 - Angular and spatial resolution imaging a single point – idealised (zero-width) microscope beamlines in green, extremes shown as well as neutral position (centred) beam. Note that grey outlines are included to illustrate the positions of lens focal points, not beampaths.

$\theta^{x,y} = \pm \theta_{max}^{xy}$ (angles translate to scanning x or y, depth is not considered) Thus an infinity corrected system was built for the microscope block of the system. It can be noted that there is no difference between the beampath of the excitation light and the fluorescence light *being imaged* (fluorescence is radiated isotropically by the chromophores), in this case. The excitation and imaging system is single sided. In figure 2.1 excitation light is right propagating whereas fluorescence being imaged is left propagating along the same green lines (do note that the green lines do only represent beampaths of the extremes and neutral position of a scan showing idealised beams, with no thickness and should not be interpreted as outlines of a real beam). See also figure 2.8.

Knowing the size of the objects being imaged allows for a very useful simplification, further elaborated in section 2.7, assuming the imaging of a point source.

2.2 Resolution

Ideally, the optical components of the system are all diffraction limited and the illuminated volume in a confocal setup is thus described by the *Point Spread Function, PSF* which is the impulse response of an optical system [2][3] and also [20]. If the light

source can be modelled as a point source the resolution of an optical system will be simplistically described by adjacent Airy discs. When the Rayleigh criterion is met for two adjacent point sources, it means that the centre maximum of the pattern produced by one source is no closer than first minimum of the closest adjacent pattern [2].

In the described system: two neighbouring molecules fluorescing can be considered point sources as their size is no more than a few nanometers long [4] whereas the fluorescence studied is in the order of several hundreds of nanometers (the visible to near infrared region when set up to use single photon processes with an excitation wavelength of 488nm). Thus the microscope can resolve the single molecules. See also the further elaboration in section 2.6.

Webb and Wilson describes in [2] and [3] the physical parameters the PSF depend on. I here mainly use Webb's nomenclature. First the *numerical aperture* is defined as:

$$\text{NA} = n \sin \vartheta \quad (1)$$

Where n is the refractive index of the medium (for air around 1, but when using e.g. oil immersion lenses the value is larger, typically around 1.4-1.5). Next the f-number is defined as:

$$f/\# = n/2\text{NA} \quad (2)$$

And in the transverse dimension (we are not interested in the axial dimension, depth, as we always stay in one plane) the x,y-coordinates are exchanged for polar coordinates and the radius r is scaled to a coordinate ρ also known as an optical unit and is designated ν by Wilson et al. The equation:

$$\rho(r) = \frac{2\pi}{\lambda} r \text{NA} = 2\pi \frac{n}{\lambda} r \sin \vartheta = r \mathbf{k}' \sin \vartheta \quad (3)$$

shows how ρ depends on the wavelength, the numeric aperture and r . Here the boldface $\mathbf{k}' = 2\pi n/\lambda$ and the ϑ is the angle as shown in figure 2.2.

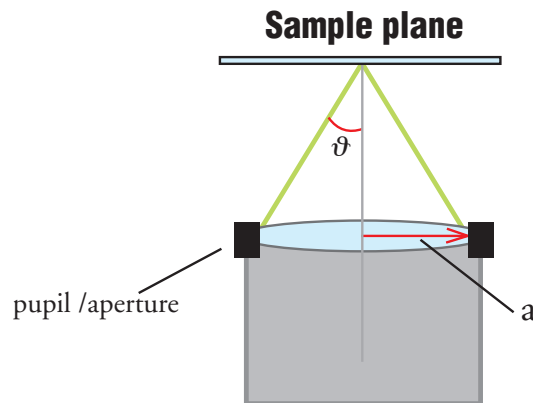


Figure 2.2 - Numerical aperture (NA), defined as: $\text{NA} = n \sin \vartheta$
The refractive index n in the formula is of the medium between the lens and the sample plane.

In theory the imaging of a point source gives rise to the image:

$$I(\rho) = \left(\frac{2J_1(\rho)}{\rho} \right)^2 \quad (4)$$

which is the PSF in the transversal plane (further elaboration can be found in Appendix A, in T. Wilson's book *Confocal Microscopy* [2] and Smith [20]). J_1 is a first order Bessel function of the first kind. It can here be seen that this is a squared sinc function (see figure 2.3a) and the familiar Airy pattern is the rotational symmetric expansion of this to three dimensions. As long as one single point is imaged through an objective lens this is true. In the case of a confocal microscope there is another imaging lens in the system, (called *Collector lens* by Wilson) and the resulting image is:

$$I(\rho) = \left(\frac{2J_1(\rho)}{\rho} \right)^4 \quad (5)$$

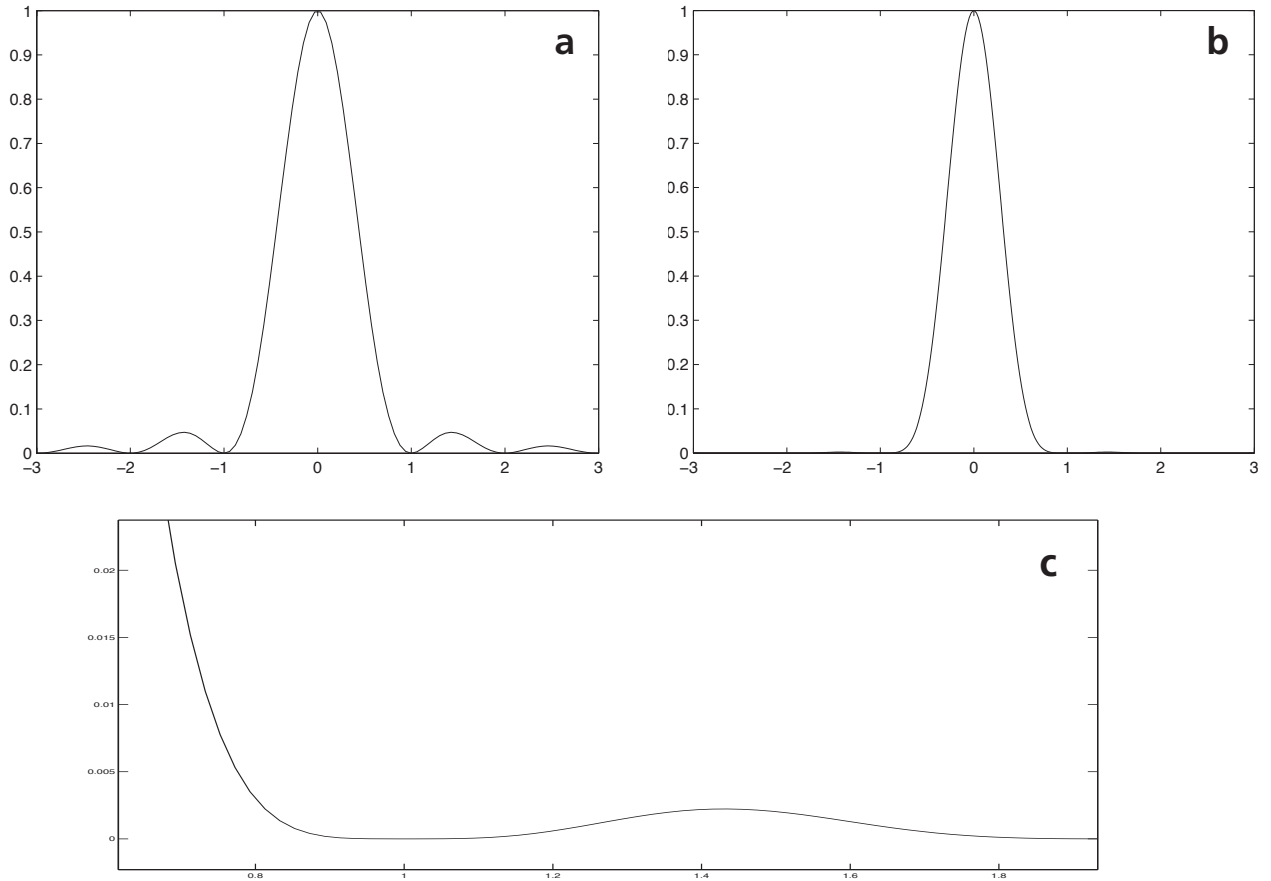


Figure 2.3 - Airy patterns, a - squared sinc, b - sinc to the fourth power, c - detail of b. The y-axis values are normalised and the unit on the x-axis is arbitrary, here it counts the dark fringes.

I.e the same function but to the power of 4 instead of 2. This is shown in figure 2.3b and it is obvious how the first bright fringe outside the first dark fringe is much weaker than was the case with only a squared function. A detail of these fringes is showed in 2.3c. This leads to the expression for the minimal diameter of the focused beam (6):

$$d_{min} = \frac{1.22 \cdot \lambda}{NA} \quad (6)$$

In figure 2.4 a real world pattern is shown. This image is a reflection off the sample glass projected onto a CCD cell used in one of the focusing strategies tried out during construction (further elaboration of different focusing strategies are found in section 3). The image has passed the microscope twice so the pattern should be according to (5), the sinc to the power of 4.

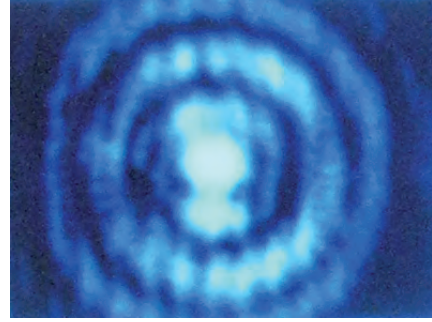


Figure 2.4 - Real world Airy pattern. Acquired by a focusing aid during construction. See also figure 3.4.

The situation for two adjacent patterns is shown in figure 2.5. Here the sum of two equally bright squared sines is shown. The top two plots shows what should be considered resolved according to the criterion given before, in 2.5b the border condition is met, first adjacent peak overlaps the first dark fringe. Only the two signals and their true sum is represented. Another way of visualising this is done in figure 2.6. In this stylised image the central peak of the intensity distribution is represented by a point and the first dark fringe is represented by a concentric circle. This does unfortunately not cover everything in a real world situation. When dealing with real signals noise is added throughout the process. In figure 2.7 different amount of random noise is added to the same type of signals and this noisy sum is plotted (note, the two intensities are equal here as well). It can easily be seen that noise is more of a limiting factor than the resolution criterion.

Webb uses the term relative intensity contrast, C , to describe the relationship between the brightest intensity, called n_b below, and the darkest intensity, n_d . The average of these is given by $2n_{avg} = n_b + n_d$.

$$C = \frac{n_b - n_d}{2n_{avg}} \quad (7)$$

The reasoning in the following centres around the fact that any image can be considered divided into pixels as no detector is infinitely small [2]. Then for every pixel the quantum noise (also known as shot noise) per exposure is given by:

$$\text{noise} = \sqrt{\text{number of photons}} \quad (8)$$

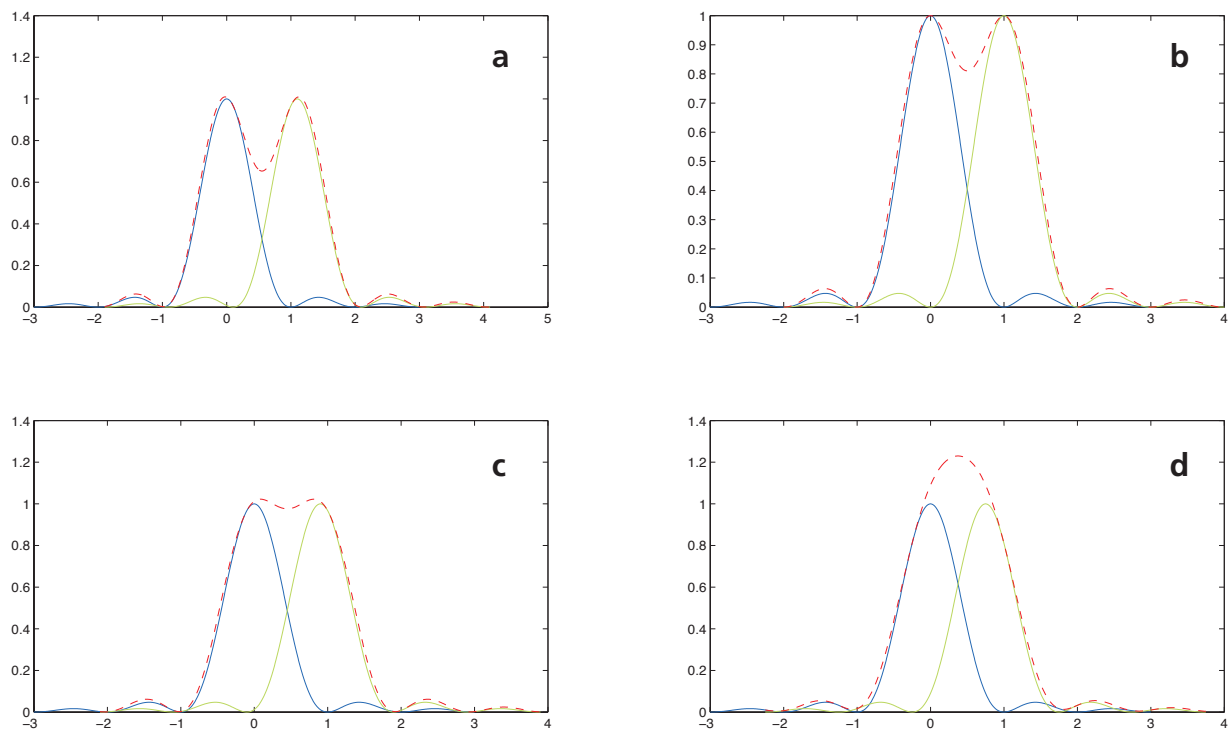


Figure 2.5 - Resolved and unresolved adjacent pixels. The Airy patterns on the top row are resolved, the ones on the bottom row are not. Red dashed line is the sum of the two overlapping intensities.

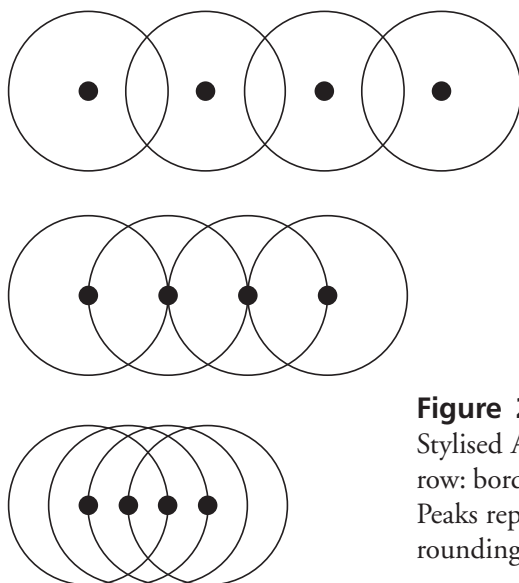


Figure 2.6 - Resolved and unresolved adjacent pixels. Stylised Airy patterns - On the top row: resolved, middle row: border condition and finally bottom row: unresolved. Peaks represented by points and first dark fringe by a surrounding circle

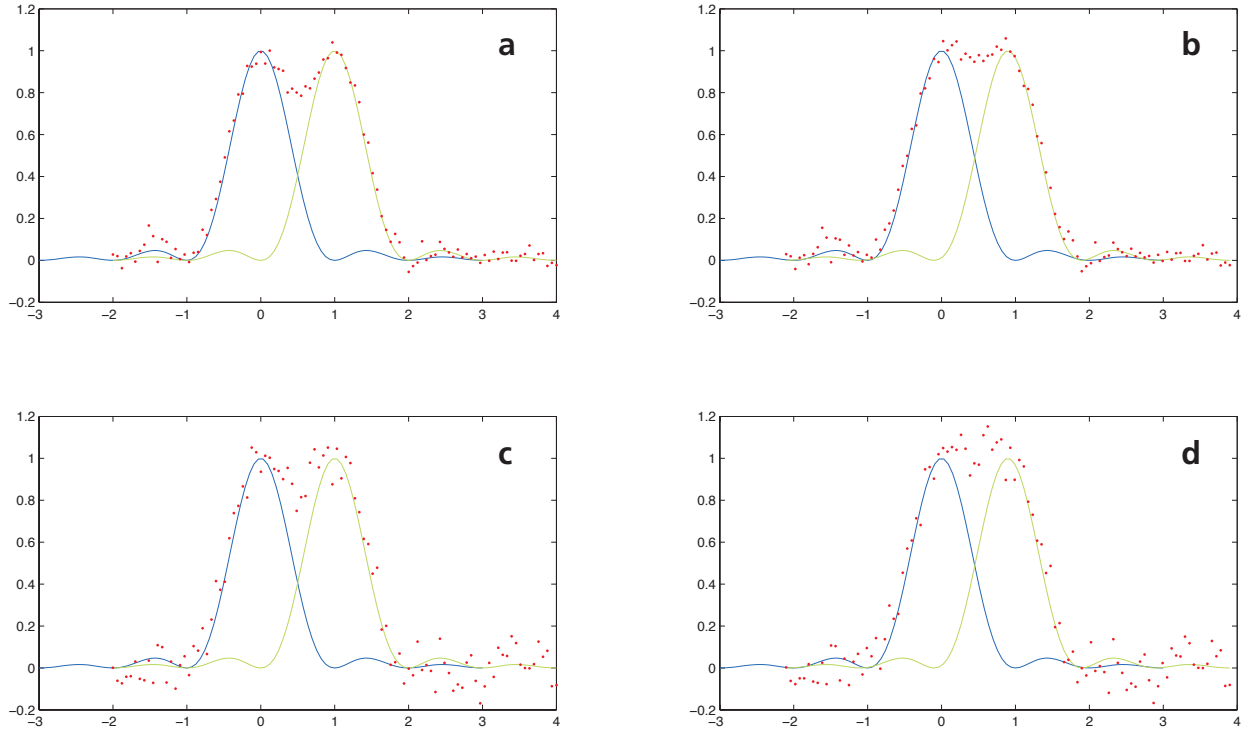


Figure 2.7 - Resolved and unresolved adjacent pixels with noisy signal. The patterns in the left column are resolved, the ones in the right are not. The red points is the sum of the two overlapping intensities with added noise. The bottom row has more noise than the upper row. Notice how it is difficult to discern the two peaks in c.

The signal to noise ratio is then (number of photons: N):

$$\text{SNR} = \frac{N}{\sqrt{N}} \quad (9)$$

This is then further elaborated to get the signal to noise ratio for a pixel. Using that the signal is proportional to the difference $n_b - n_d$ and that the noise is proportional to the square root of n_{avg} the signal to noise ratio for a pixel can be written:

$$\text{SNR} = \frac{n_b - n_d}{\sqrt{n_{\text{avg}}}} = C \sqrt{n_{\text{avg}}} \quad (10)$$

If we assume that present background light is below the noise floor the signal is directly proportional to the intensity n_b and the intensity n_d is directly proportional to the dark count of the sensor.

The sources of noise are distributed along the path from the sample to the software. In this discussion the main focus is on the detection components, including quantum and thermal noise.

2.3 Sensitivity

In the setup described in this thesis an *Avalanche Photodiode* (APD) was used as a detector. The dynamic range of the device used is from its noise floor being TTL pulses at a rate of about 250 pulses per second at complete darkness (measured value), to a few million pulses per second when saturated. The noise floor, caused by quantum, thermal and electronic noise is called the *Dark Level*, *Dark Level Count* or simply *Dark Count*. The noise floor determines the practical minimum amount of light needed to register a change in intensity. An APD can theoretically register single photons. The pulse created by single photons are drowned in the background noise if they come at a rate more seldom than 250 photons per second. To lower the noise floor the silicon die is cooled to low temperatures, to lower the thermal noise. In a practical application the 250 counts per second turns out to be manageable using active cooling with Peltier elements. This is where the confocal scheme proves very useful, since the interference from fluorescence and other light sources outside the objective lens' focal plane is effectively blocked by the pinhole. More details on the physical design in section 3 *Implementation - Hardware*.

An alternative to an APD detector could have been a Photo Multiplier Tube. A PMT can more easily be cooled to lower temperatures and have lower thermal noise at about the same quantum efficiency but such a sensor can be much more costly and is bulkier. The light sensitive area is generally much larger than on the APD thus reducing the need for the extra focusing lens in the detector line. The APD has an active spot with a diameter of roughly 100 μm , That is why additional optics is needed after the pinhole in the detector line to focus the collected light onto the APD's active area (see also the schematics in figures 1.2 and 3.1). This small active area is also positive as it acts as a second pinhole in the system

2.4 Angular and Spatial Resolution

The system, ideally being diffraction limited, can focus a laser beam with a wavelength of 488 nm to a interference pattern with a minimum diameter of the Airy disc of about 0.5-1 μm (depending on NA, see (6)). Moreover specifically equations (4) applies for the illumination and for the imaging equation (5) applies. The excitation light is projected to this minimal size, obtained when the system is perfectly focused, on to the sample of the matter intended for analysis.

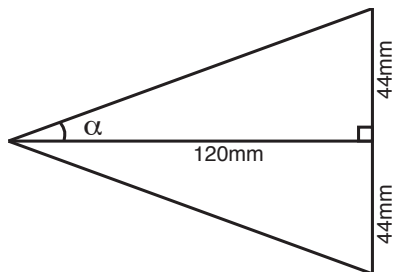


Figure 2.7 - Geometry of the trigonometric calibration of mirror angles

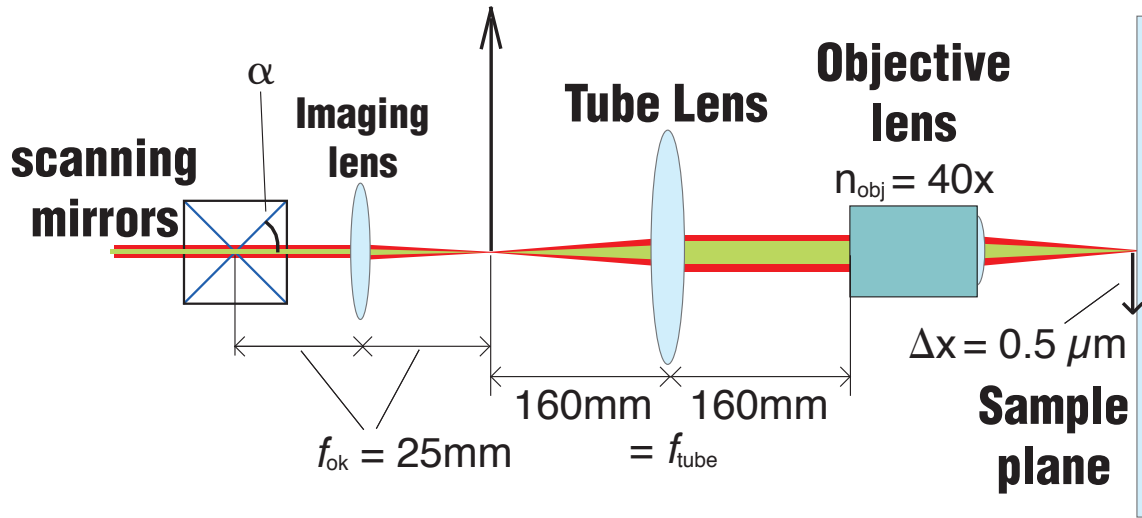


Figure 2.8 - How to convert the scan angle to a distance at the sample plane. Using a fixed Δx set to e.g. $0.5 \mu\text{m}$ the required mirror angle for one pixel can be calculated. For the values in the image $\alpha = 0.5 \mu\text{m} \cdot 40 / 25 \text{ mm} = 0.8 \cdot 10^{-3}$ radians per pixel. Beam thickness is illustrated for a diffraction limited system, green outline = excitation light, red outline = fluorescence light

$$\alpha = \frac{\Delta x \cdot n_{\text{obj}}}{f_{\text{ok}}} \quad (11)$$

The galvanometers controlling the scanning mirrors have limited resolution. The mirror position is addressed with a signed 16-bit integer meaning that the full angular range ($\pm 0.3514 \text{ rad} \approx 20.14^\circ$) from the neutral position, is 32768 steps in either direction, i.e. every addressable step of the galvanometers corresponds to $62.9 \cdot 10^{-3}^\circ$. The requested angular position is not fully stationary either, it has a slight noise, it vibrates slightly, corresponding to a few steps, due to the environment: electric noise, air streams and RF interference. During testing it was discovered that the field surrounding a GSM transmitter did in fact induce an audible rattle in the galvanometers even in the far field (more than around 70 cm for a 900 MHz transmitter [6]).

Using an objective lens with n_{obj} times magnification the angles scale down according to formula (11). Specifics are shown in image 2.8. A choice of a 40x magnification gives a mirror angle difference of $0.8 \cdot 10^{-3}$ radians per pixel

The full angular range of the mirrors was measured to have a true value to work with when building the actual setup. A simple trigonometric method was used. A fixed adjacent at 120 mm was used, the mirrors was placed in the corner and at the opposite, a screen was placed and using a laser the full range was marked and measured to $\pm 44 \text{ mm}$.

For the case in figure 2.8 with every pixel is assumed to be $0.5 \mu\text{m}$ in diameter giving the value $0.8 \cdot 10^{-3}$ radians per pixel. This means that a translation of the excitation spot one pixel corresponds to 72 steps on each axis and a full range of 100 by 100 steps corresponding to an imaged area of 50 by 50 μm would be 7200 steps on each mirror, slightly less than 5° . This is about one eighth of the usable range of the mirrors.

It is desirable to keep the angles small (smaller than 10° at least) as the system then can be considered using paraxial approximations, i.e. $\sin(\theta) \approx \theta$, $\tan(\theta) \approx \theta$ and $\cos(\theta) \approx 1$ and the system does keep reasonably linear in this range.

2.5 Speckle

When using a coherent light source such as a laser in a wide-field microscope setup, speckle patterns can make the image hard to use unless imaging fluorescence [2], due to small variations in topology of the sample in the order of multiples of halves of the illumination wavelength. These minute changes causes a pattern of intermittent constructive and destructive interference of the reflected light, seen as the familiar “grainy” look of laser spots. Compare with the discussion of wide-field microscopy in section 2.1, see also figure 1.1. Using fluorescence and a confocal strategy the speckle problem can be disregarded for two reasons:

1. Fluorescence is usually not coherent (some dyes can theoretically lase, but that can be disregarded in the absence of a suitable cavity) and as long as the excitation light is appropriately filtered out from the signal, no problems should arise due to speckle.
2. The single point/single plane discrimination removes speckle problematics automatically as the only point reflecting light is the presently illuminated spot and no matter what interference the incoming light displays it should be consistent over the imaged spot at least and every point on the full image scale is normalised.

Finally, as single molecules are imaged there should be no coherent light in the fluorescence, all molecules are located in the same plane and if an oil immersion lens is used, most of the surfaces that could potentially give rise to a speckle pattern have a refractive index very close to the oils and the phenomenon is even further mitigated. The

2.6 The Dose Concept

In this paper the concept of *dose* and *imaging dose* is introduced. This term was chosen due to the similarity of the situation where biological matter is exposed to different types of harmful ionizing radiation. As has been noted before, some compounds are inherently sensitive to light and can easily become subject to photo bleaching. The exposure to light in this case is the cause of degradation so this same concept is applied to the described imaging systems.

The dose is chosen to describe the product of optical power and illumination time per unit area (equation (12) below). This gives the available energy that can be absorbed. More generally the concept should be per volume unit, but in this case only planar samples with single molecule detection is considered so the simplification to area is done. In the confocal case the assumption is made that the microscope focuses the light diffraction limited to a point having a wavelength dependency as shown in (4).

$$D_{Img} = \frac{P_{opt} \cdot t_{exp}}{A} \text{ J/cm}^2 \quad (12)$$

Further we also consider the apparent size of the molecules imaged being determined by their absorption cross section, σ , which is wavelength dependant [15], the concept can be even further simplified to a per molecule dose which is identical in both the widefield and the confocal scheme. The light emitted (isotropically) by the molecule is proportional to the energy it absorbs. Thus the same excitation light power density and the same exposure time are required to get a certain signal strength (a certain number of APD pulses per time unit).

That the dose actually is *per molecule* is provided that the molecules really are singularly distributed in the sample (nonradiative energy transfer between adjacent molecules is possible).

The P_{opt}/A is the energy concentration of the illuminated area. In the case of confocal microscopy of single molecules it is assumed that all energy in an area corresponding to the molecules absorption cross section within the diffraction limited spot is available for the molecule. In a comparative wide-field case, the energy concentration of the total illuminated area (in the calculations done here, this area is chosen to be the same as the scanned area in the confocal case) is assumed to shine on a set of more than fully resolved molecules as described in section 2.3. The calculation is done assuming Airy patterns bordering each other in a matrix with their first minima overlapping. In the confocal scheme, the excitation spot is swept over the whole area so the mean dose per line should be equal. The value is then applied per molecule.

One benefit of the scanning method is that a molecule receiving a specific dose will receive it in portions, with a time for relaxing in between illuminations. The time between exposures is determined by the *reset time* (set beforehand, a value of 75ms was used during testing) and the *sweep time* (the resolution in pixels times the exposure time).

During some the experiments performed in the project the optical power at the sample plane was measured to between 0.15 μW and 0.8 μW . A very simplified illuminated area is just taking the square of the illuminated area. Light at 488 nm was used so the smallest theoretically possible point is roughly 1 μm ($=1 \cdot 10^{-4}$ cm) giving an area of about $1 \cdot 10^{-8}$ cm^2 . This value for a lens with NA=0.6 is found using (6).

$$d_{min} = 1.22 \cdot 488 / 0.6 = 992 \text{ nm}$$

So in this case the power density is: $0.15 \cdot 10^{-6} / 1 \cdot 10^{-8} \approx 15 \text{ W/cm}^2$. In the case of the higher input power we get around 80 W/cm^2 . Also, if the NA allows it, the focal point can be smaller, resulting in higher power density. The dose is then determined by the exposure time and the absorption cross section. These power densities are, as noted above, the same as the ones that has been used in earlier research using wide-field illumination. During some experiments that exhibited fluorescence blinking, conducted by Mirzov & al. and presented in [4-III] typical excitation power densities were in the order of 4-15 W/cm^2 , whilst in others they were significantly higher (e.g. $\sim 300 \text{ W/cm}^2$ in [4-II,IV]).

Here the crucial point can be seen. The total dose will be lower in the confocal scheme. The exposure time per point, and thus also the dose, to the sample will be the same if the power density is the same. But there is a difference. When analysing specific

points on the sample or when focusing the microscope only a single point will be hit (and bleached if it has a molecule on it) whilst the rest of the sample is left intact. Thus consecutive measurements can be made on different molecules in the same sample even if the first molecule is destroyed. This would not be possible in a wide-field scheme where the whole sample would have been illuminated during the measurements on the first molecule.

2.7 Simplifications

The system described here is designed for imaging of single molecules. More specifically, building a system for analysing the properties of certain organic polymers was the main objective of this project.

One type of polymer that has been studied fairly extensively at the Department of Chemical Physics is a vinyl called MEH-PPV. It was chosen as a prototype sample for the trials of the built system.

The monomers of the polymer have the structure showed in fig. 2.9. The research conducted at Chemical Physics has generally not been directed at studying the monomers but polymers of different (often significant) lengths. It has been shown that the spectroscopic properties of MEH-PPV molecules depends on how straight chains of monomers (typical size is in the order of 5 monomers), act as (semi-) independent chromophores [4][5]. The sections of the polymer chain that interact optically are referred to as *spectroscopic units* by Mirzov & al. (nomenclature taken from [4]). These semi-independent units can absorb and fluoresce independently, but there exists possibilities to transfer absorbed energy along the chain of spectroscopic units non-radiatively. This results amongst other things in a peculiar “blinking” effect, fluctuations in fluorescence intensity when exciting single MEH-PPV molecules with CW light [4], as the energy being transferred along the chain sometimes ends up in “dark states” such as e.g. the first excited triplet state which cannot relax to ground state radiatively. This can be due to internal (such as the triplet states mentioned above) or external quenching (consult e.g. Thomson’s and Mirzov’s work for further elaborations on the properties of such polymers).

It was shown during the course of the project that it was indeed possible to see the single molecules, and also the blinking effect using the constructed confocal instrument. The difference to the earlier wide-field strategy used by Mirzov is that due to the scanning of the sample illumination is intermittent. The phenomenon was nonetheless visible and showed up as much darker lines on the intensity graph making up the image. In image 2.9 such a recording is shown. Note that the pixels cannot be considered resolved at this magnification, the large size of the luminous spot must be considered an

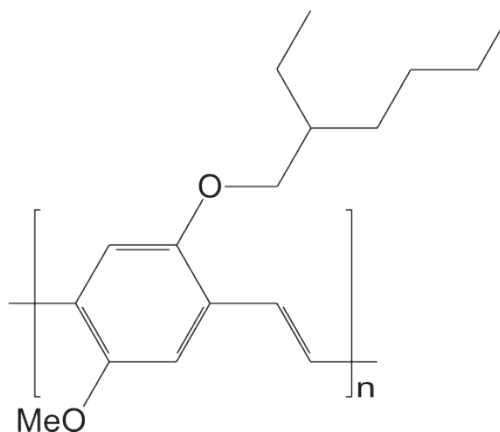


Figure 2.9 - MEH-PPV polymer

image of the PSF as every pixel is far below resolvable sizes. Consider instead each row as a time resolved vector, as every pixel in the image is captured at different occasions during the scan.

The spectroscopic units, the optically active parts of the molecules studied in this case, are no longer than a few monomers each and the whole molecules can be considered point sources as their size is at least an order of magnitude smaller than any wavelength in the visible optical regime. An MEH-PPV chain of 100000 units has a length of only around 230 nm but the chain will be collapsed into a nano particle with a diameter of a few nanometers [4-IV].

It can also be noted that the energy transfers between spectroscopic units will in the majority of cases be confined to the small bundle so the point of origin of the electromagnetic wave will not shift significantly regardless of radiating unit. It can within reason be considered unlikely that transferred energy would move distances in the order of fluorescence wavelengths at which the movement could theoretically be detected.

2.8 Spectroscopic Considerations

To adapt the system to different species and excitation wavelengths, the main method is to switch laser line at the source in conjunction with an exchange of the dichroic mirror and applicable cleanup filters. The system as it is described in this report is designed only for redshifted fluorescence, see section 6 on further development for a discussion of possible designs for using blueshifted fluorescence, two-photon processes and so on.

The linewidth of the excitation light can be tuned for the analysis at hand by the choice of laser, in this project the 488 nm line of a multiline Ar⁺-ion-laser was used, and adjusting its working parameters. The second part that needs to be adjusted is the dichroic filter cube that will need to be selected accordingly. For simplicity and consistency Olympus prefabricated filter cubes for fluorescence microscopy was used. The filter cubes contains a fixed dichroic mirror and two detachable cleanup filters, one for incoming excitation light (reflected by the mirror) and one for fluorescence light (transmitted). Depending on the situation different combinations can be used. Two main filter type combinations are available from Olympus, one with the cleanup filters both being passband filters allowing for a wavelength selectivity in the fluorescence path, the other combination is using the same excitation passband filter and the same dichroic but using a lowpass filter in the fluorescence path. In figure 2.10 two typical filter cube combinations are shown (data taken from Olympus [10]). As can be seen from figure 2.10b the difference between the transmittance of the dichroic and the lowpass filter is that the lowpass filter has a steeper edge. The use of a setup with dual passbands with the fluorescence filter on a flipper could give approximately the same result as the lowpass cube when the flipper is down. The prefabricated cubes with passband filters come with either a narrow passband or a slightly wider passband *on the excitation side* (the cubes are designed for use with incoherent light sources as well). The difference can be seen in the comparison between figure 2.10a and 2.10c.

Using a passband only cube one get a spectral selectivity corresponding to the passband of the filter. As the filters are dismounted from the cubes used in this setup

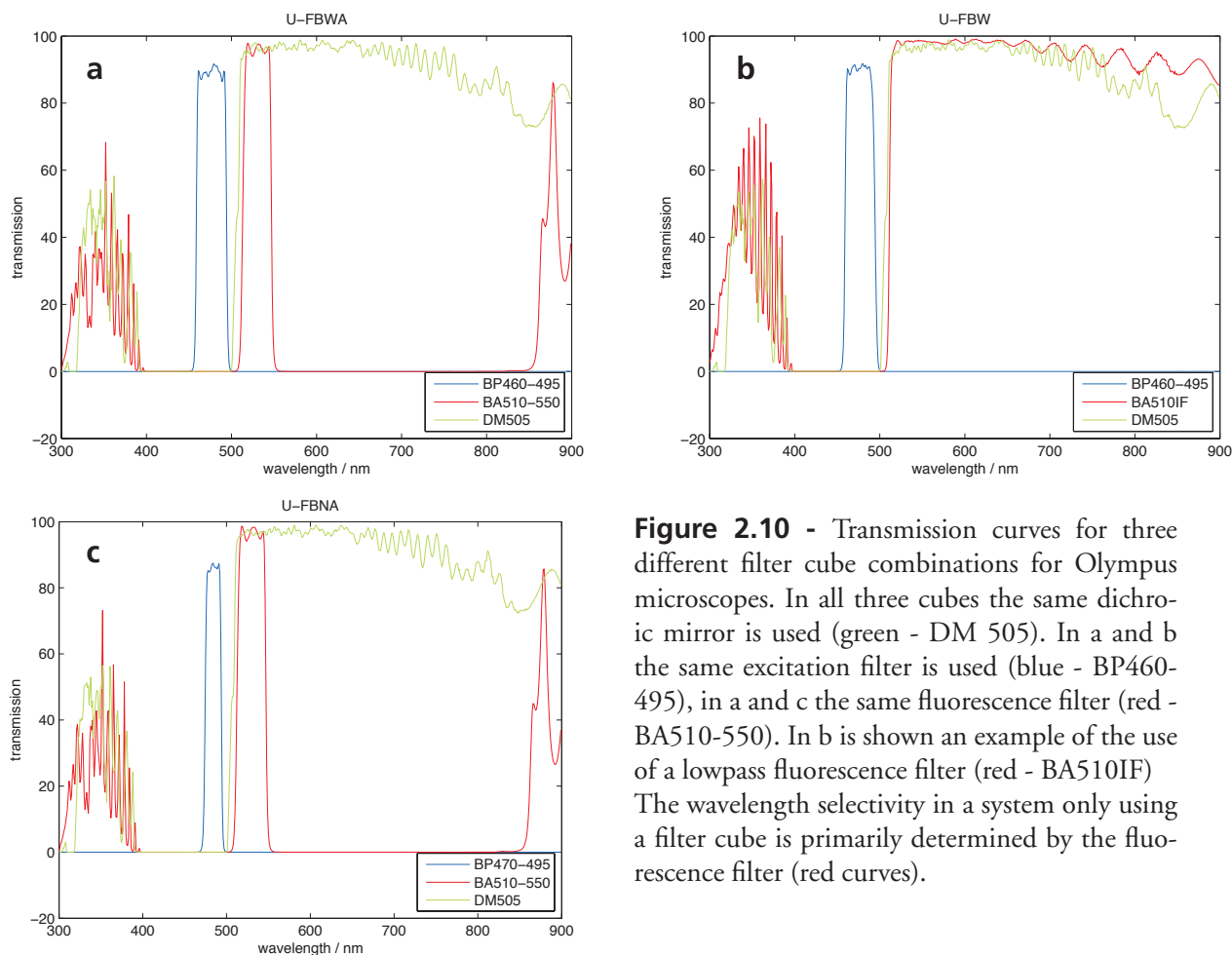


Figure 2.10 - Transmission curves for three different filter cube combinations for Olympus microscopes. In all three cubes the same dichroic mirror is used (green - DM 505). In a and b the same excitation filter is used (blue - BP460-495), in a and c the same fluorescence filter (red - BA510-550). In b is shown an example of the use of a lowpass fluorescence filter (red - BA510IF). The wavelength selectivity in a system only using a filter cube is primarily determined by the fluorescence filter (red curves).

they can easily be exchanged for different wavelength ranges while retaining the present cube's mirror. The signals from single molecules are weak as can be seen in section 5 and figure 4.2 and at the moment it may not be possible to use any higher resolution spectroscopic methods, but earlier experiments have shown the quantum yield should be sufficient and suggest that such matters as alignment issues explain the relatively low sensitivity. With the sensitivity issues solved, methods to increase spectral selectivity include multiple detectors served by daisy-chained dichroics or gratings. See also section 6 - *Further Development*.

In the constructed setup, no consideration has been taken to polarisation, neither on the excitation side nor in the retracing fluorescence path. It can easily be introduced as needed.

For the MEH-PPV used as a prototype molecule during live testing Mirzov presents in [4] a model absorption spectrum. There is shown an absorption cross section for different polymer chain lengths measured in monomer units. An example of the results Mirzov found is single molecule fluorescence at between 16000 to 17600 cm^{-1} (568 - 625 nm) with 514 nm excitation [4-II].

3. Implementation – Hardware

The setup can be divided into a set of distinct interconnected blocks. Schematic 3.1 describes the physical parts. In the schematic green colour shows the beampath of the excitation light and red colour shows the beampath of the fluorescence. The cleanup filter marked in the excitation light beampath is designed to make sure the excitation light is cut off at a proper wavelength. E.g. when using incoherent light sources such as

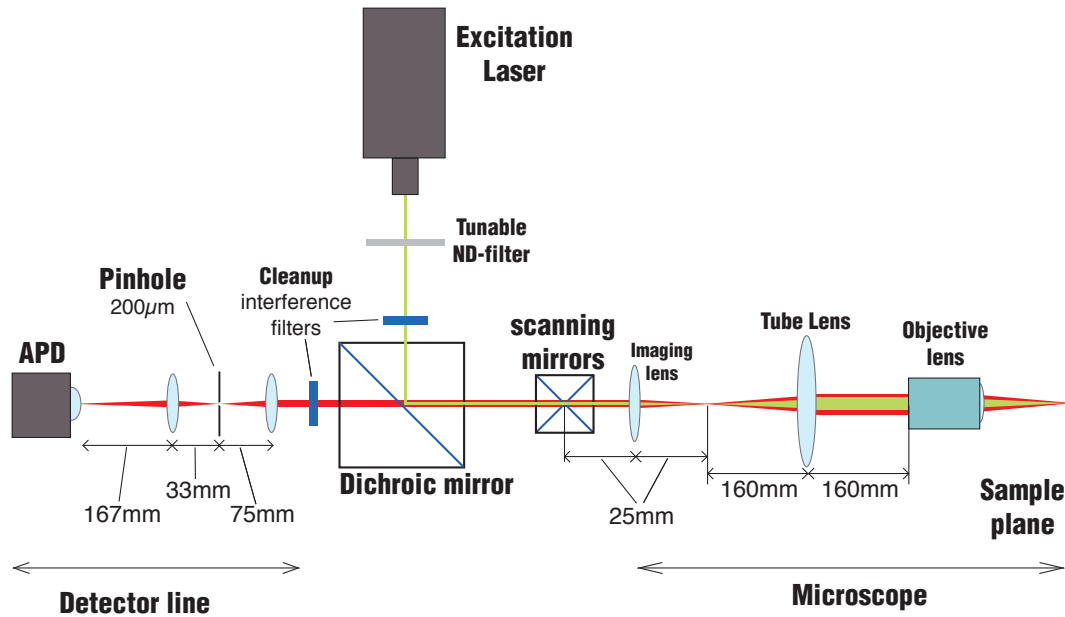


Figure 3.1 Schematic describing the basic layout of the microscope setup

gas discharge lamps in wide-field fluorescence microscopy it is used to make sure only interesting wavelengths, sufficiently short (wavelengths shorter than the dichroic's pass-band) are passed to the dichroic mirror. It is well worth noting that although not strictly necessary it has a function as the Argon ion laser used in the project has multiple lines and this filter still makes sure none of the longer lines inadvertently interfere with measurements and other stray light is blocked.

The filter found after the dichroic in the detector line is used to make sure leaking reflected excitation light arriving with the fluorescence from the sample is removed so that it does not interfere with measurements. The dichroic could possibly let some of the reflected excitation light through and cause a raising of the noise floor at the APD (or in extreme cases, even saturation of the APD). This feature is used in a focusing strategy. This filter is mounted on a flip mount, so when focusing the transmitted excitation light reflected from the sample plane is used as an alignment aid. The filter is removed and the primary aligning of the beam through the pinhole can be done. Fine tuning is then done by moving the APD. More on this later.

3.1 The Microscope Building Blocks

The following is a further description of the blocks making up the whole system:

- **First block:** The excitation light is passed through an inverted beam expander (not shown explicitly in the schematic but visible in figure 3.2) placed after the laser and a tunable neutral density filter. This reduces the beam diameter and thus reducing the amount of light spillage. It also make sure we keep the point source nature of the incoming beam. It is desirable to pass as much of the light through the aperture of the objective lens as possible as light hitting construction details firstly does nothing useful and secondly the detail might fluoresce, adding noise to the data. A ND-filter is used to regulate the light intensity at the sample. Especially with a laser operating in CW-mode it is desirable to keep the power down in order not to damage the sample. It is a complement to the power settings on the laser itself.

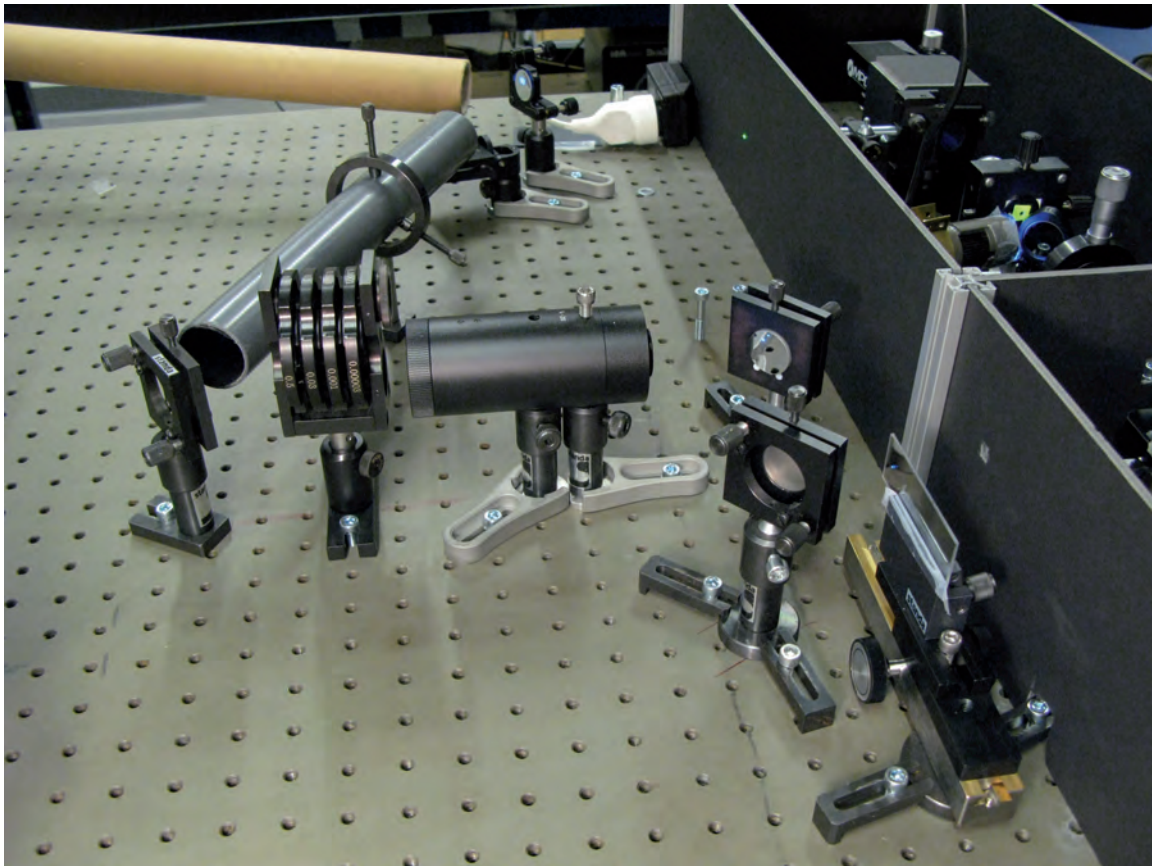


Figure 3.2 - Beam transport - The laser is passed through the tubes, used to counter disturbances due to air flows etc. In the middle there is a ND-filter revolver and the tube after that is the inverted beam expander. On the translation stage, on the right just before the beam enters the box, a second tunable ND-filter is mounted.

- **Second**, the light is reflected by a dichroic mirror (figure 3.1) into the main beampath of the microscope. The beam passes through the two diaphragms placed between the dichroic mirror and the scanning mirrors. These diaphragms are primarily used as alignment and focusing aids (realignment is needed quite frequently due to the sensitivity of the setup). After the diaphragms the beam is passed into the scanning mirror setup. The two scanning mirrors are mounted on precision galvanometers mounted at a 90° angle and provides the X-Y scanning needed.

- **Third**, the excitation light is passed through the infinity corrected microscope (see schematic in figure 3.1) and the fluorescence light retracing exactly along the same path as the excitation light will pass through the dichroic mirror and into the...

- **Fourth part**, the Detector Line, which consists of a lens (focal length chosen to 75 mm) focusing the parallel light through a 200 µm pinhole (experiments using a 100 µm pinhole where performed, but it proved very difficult to focus light onto a hole of a size on the edge of what the human eye can resolve unaided), and thereafter another lens (focal length chosen to 100 mm and mounted offset as shown in figure 3.1) is projecting an image of the pinhole onto the actual detector, in this case being an Avalanche Photo Diode (APD). This last lens is needed since the sensitive area of the APD is very small, only roughly 100 µm across.

- **Fifth**, the TTL pulses from the APD are counted by a PCI DAQ-card from National Instruments. This is mounted in the same computer that is controlling the scanning mirrors through a hardware controller attached to a standard serial interface.

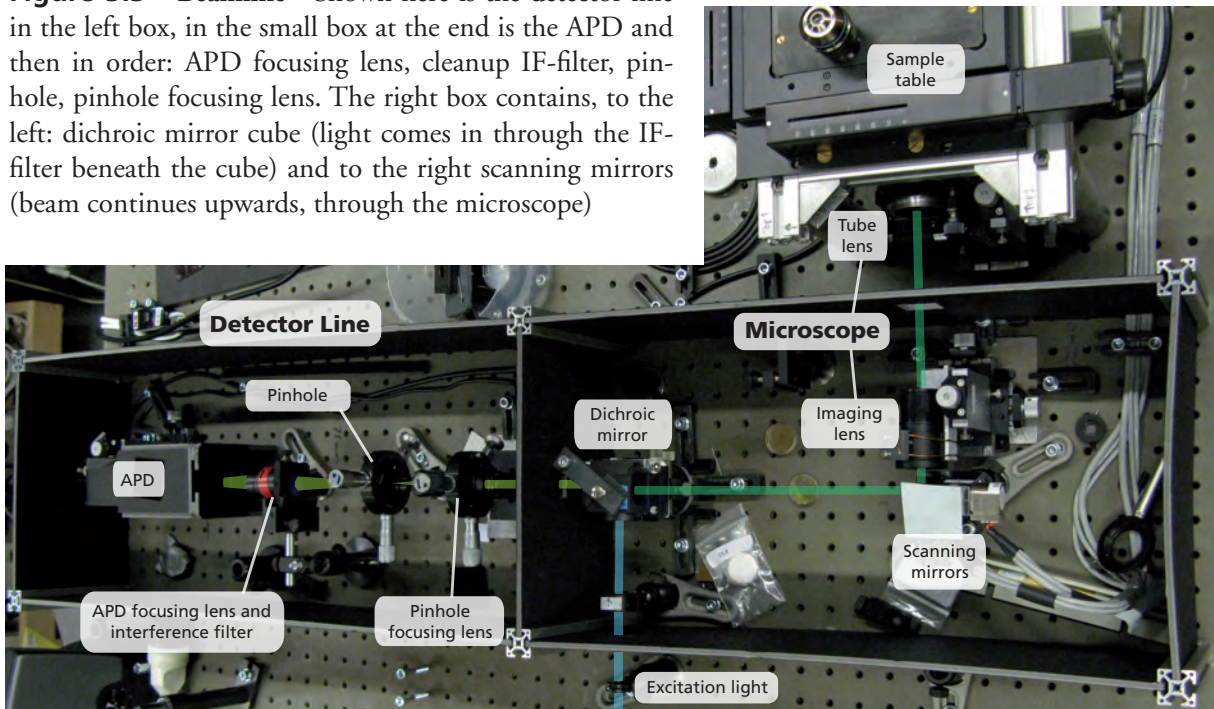
- **Sixth**, a control interface written in LabVIEW is used to operate the microscope and do some basic data analysis. Furthermore due to the controllable sync pins on the scan controller an interface to control an electric shutter is provided for convenience. Further controllable features can easily be added as needed later.

The system is quite long, as can be seen in figure 3.3, as the beamlines deliberately has been kept as unconvoluted as possible. The longest axis is the detector line and the beampath to the mirrors, the total length of this beampath is around one metre.

The setup has been built around a XY-translation board acting as a sample plane with a standard microscope stand with focusing screws and an objective lens holder fixed underneath. As shown in figure 3.4 the sample plane is horizontal and the objective lens is placed directly below it.

The microscope is basically an infinity corrected microscope with a 45° mirror bending the beampath in the infinity portion, between the objective and tube lenses, 90°. A standard eyepiece is fitted to the system with the scanning mirrors placed at its focal point.

Figure 3.3 - Beamline - Shown here is the detector line in the left box, in the small box at the end is the APD and then in order: APD focusing lens, cleanup IF-filter, pinhole, pinhole focusing lens. The right box contains, to the left: dichroic mirror cube (light comes in through the IF-filter beneath the cube) and to the right scanning mirrors (beam continues upwards, through the microscope)



3.2 Aligning

In order for the images to be consistent it is important to at least make sure that the sample plane and the objective lens are parallel and perpendicular to the optical axis. This was accomplished by passing an alignment laser through the microscope with mirrors mounted and centred but no lenses mounted. Using a prototype pole to ensure that the optical axis was level until the 45° mirror and a plumb-bob to ensure that the optical axis was perfectly vertical after the 45° mirror the full length of the optical axis from the scanning mirrors to the sample plain was set. After this the sample plane was made parallel to the objective lens fastening plane by use of mirrors placed at the respective planes making sure that the reflection hit the same spot.

After this the lenses were introduced and the integrity of the optical axis was ensured and the initial focusing of these parts could be done.

Later it was discovered that the initial mounting of the dichroic mirror on an adjustable table was too sensitive to vibrations. It was not possible to adjust the component with high enough accuracy. This prompted the introduction of two mirrors to perform the same task as the adjustable table did and the mounting of the dichroic mirror on a fixed table. This construction can be seen in figure 3.2. After a realigning of the optical axis the detector line could be constructed.

When aligning this part a mirror is placed at the sample plane and the cleanup interference filters normally used in connection with the dichroic mirror to get as clean a signal as possible, are removed and the light leaking through the dichroic mirror is used. In the detector line a fixed lens is mounted to focus an image of the pinhole onto the APD. The pinhole (the pinhole is adjustable on the XY-axes) and the APD (the APD is also mounted on a three-dimensional stage, adjustable on the XYZ-axes) is adjusted to this scheme while maintaining as precisely as possible the desired optical axis height

(using a prototype pole like before). Finally the first lens, the one focusing the incoming light to the pinhole, is mounted and used to make sure that the light from the appropriate plane passes through the pinhole.

Making sure that the focal plane of the first lens is located at the pinhole turned out to be somewhat tricky. An initial mistake was made, focusing *all* reflected light instead of just the light from the single plane that was to be imaged, effectively removing the depth of field advantage of a confocal setup. It was actually possible to image extremely bright chromophores even with this type of misalignment, making it difficult to spot. Fluorescent beads were used during these tests and their quantum yield was extremely good so it took quite some time before the error was found. That something wasn't right could be seen as the SNR was very bad for a real sample. Later when the error was found by checking all physical dimensions of the setup it was realised what the spot appearance should be like. Rather than a single bright spot it should be a larger bright area with a concentrated bright spot in the centre, being the sample plane. (It is implied that the microscope actually is focused to the desired sample plane. In the case of alignment work, being the mirror surface.)

3.3 Focusing

What proved to be one of the most difficult problems to cope with was to design an effective way of focusing the microscope. Since at no time the whole sample is imaged simultaneously it is not possible to view the sample through e.g. a second ocular unless a secondary, wide-field lighting scheme is used in conjunction with the excitation beam. Also, if the sample is particularly light sensitive and bleaches easily this might not be a good choice of focusing strategy. Then the "*minimally destructive*" method described below is a better choice.

Several approaches were suggested, some more practical than others.

•1 Wide-field solutions

a) A simple approach is to mount a light source illuminating the sample in a way effectively turning the microscope into a standard transmission microscope. The image can then be observed either directly by eye or by using a camera, looking through the setups eyepiece/scanning lens. A flipping mirror or a beam splitter is typically needed to access the beampath. Alternatively a beamsplitter or flipping mirror is placed so that a second ocular can be placed confocal with the actual scanning lens, i.e. the beam splitting is done in the tube parallel beam. This approach is inherently difficult to configure as the second ocular needs to be very accurately placed with respect to the scanning lens. Also, a highly diluted sample as would be the case when studying single molecule preparations. It would not necessarily show any features with enough contrast to be useful when focusing so such a primer must then be added.

b) Of course using a wide-field lighting scheme using fluorescence with excitation light illuminating the sample the same way as the system shown in figure 1.1 to find the correct focus using the same strategy as for the transmission method, is possible, but

then the whole advantage of the confocal strategy, less exposure of the sample to excitation light, smaller imaging dose, is lost in the focusing process.

•2 Single point solutions — Minimally destructive solutions

This method uses a laser beam focused on the sample, either an auxiliary laser or the actual excitation beam can be used. The beam is focused to one point on the sample exactly the same way as during scanning. This point on the sample will be “sacrificed” and the direct reflection is used for focusing.

First a rough focusing is required which is accomplished by observing the reflected light on diaphragms and on a screen mounted directly around the pinhole. When the setup is in focus a maximum of light will pass through the pinhole as per the design. A screen to be observed by the naked eye or a CCD element is therefore placed after the pinhole for finetuning. The preferred lighting strategy is to use the excitation beam since the use of an auxiliary laser is ridden with the same problems as the extra eyepiece strategy mentioned in the wide-field description above: it is very difficult to make sure the focusing beam and the excitation beam are perfectly parallel. If they are not, the reflection from the sample will almost certainly be imaged on the side of the pinhole instead of the light passing through it. When using the excitation beam for focusing, the fact that some light of the wavelength designed to be reflected actually passes through the dichroic mirror and thus can be used for focusing.

In the setup described in this paper the excitation beam version of the second solution is used. All cleanup filters are placed after the pinhole, just before the APD, and a flipping mirror is placed just after the pinhole but before the filters. The light passing through the pinhole is projected on a CCD element (see figure 3.4). Since the microscope is diffraction limited the projected pattern will be an Airy pattern and when the substrate glass surface is close to be in focus, the Airy disc projected is used to finetune the focus. A modified USB connected webcam/microscope with its optics removed is mounted inside the detector line box. The standard software accompanying the camera is used in videomode to find the best focus. A number of filters are included which can be useful when fine-tuning. Especially a 3D filter converting intensity to a pseudo-3D landscape is helpful when finding a maximum in the centre peak. Figure 2.4 shows a snapshot from this CCD.

To do the fine-tuning a proven effective method is to first do the rough focusing using the principles above and then with low intensity laser and possibly disengaged cleanup filters (the interference filters blocking reflected excitation light from reaching the APD and saturating it) monitor and maximise the readout from the APD. With low intensity laser this can be achieved and the reflected light can be used to pinpoint the samples surfaces (top and bottom), by simply finding the maxima of counts from the APD. It is important to think carefully here, to make it simple on positions the focus clearly outside on either side and then actually count the maxima while pulling focus through the sample carrier and then go back to the correct plane.

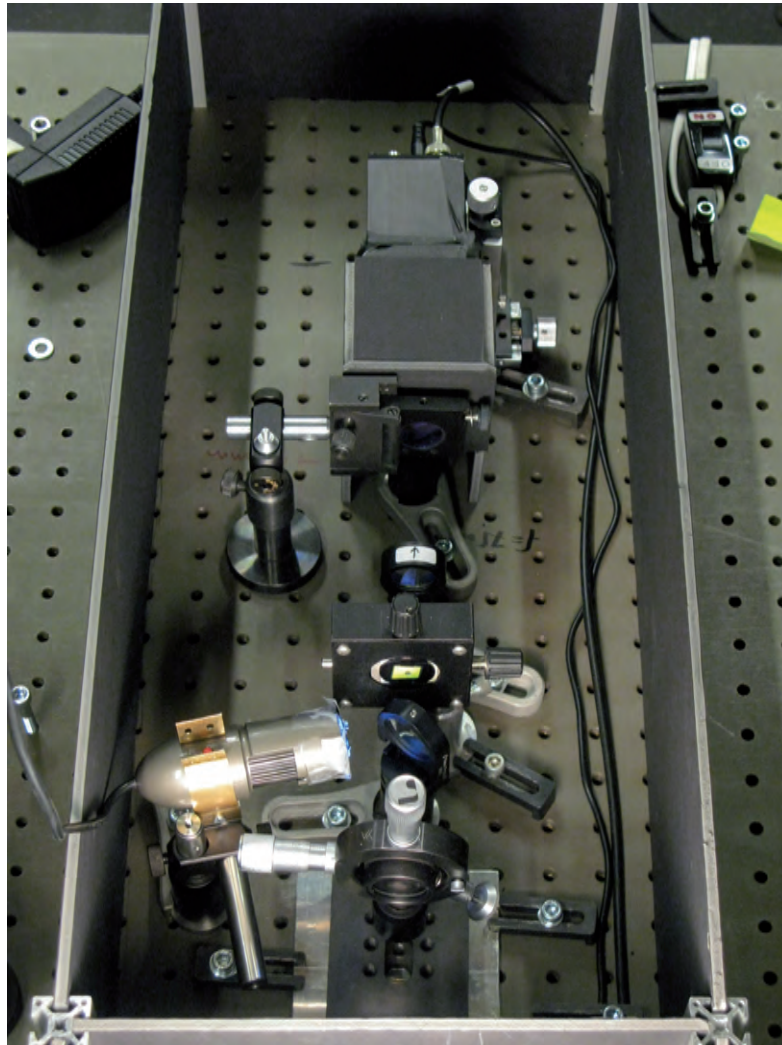


Figure 3.4 - Detector line - Shown here is the detector line with the APD at the top, next the cleanup filter and APD focusing lens. The yellow paper mounted on the pinhole is a focusing aid. The camera CCD on the left is coplanar with the pinhole, the flip mirror directly after the pinhole focusing lens directs the light to it while focusing. An image from the CCD is shown in figure 2.4.

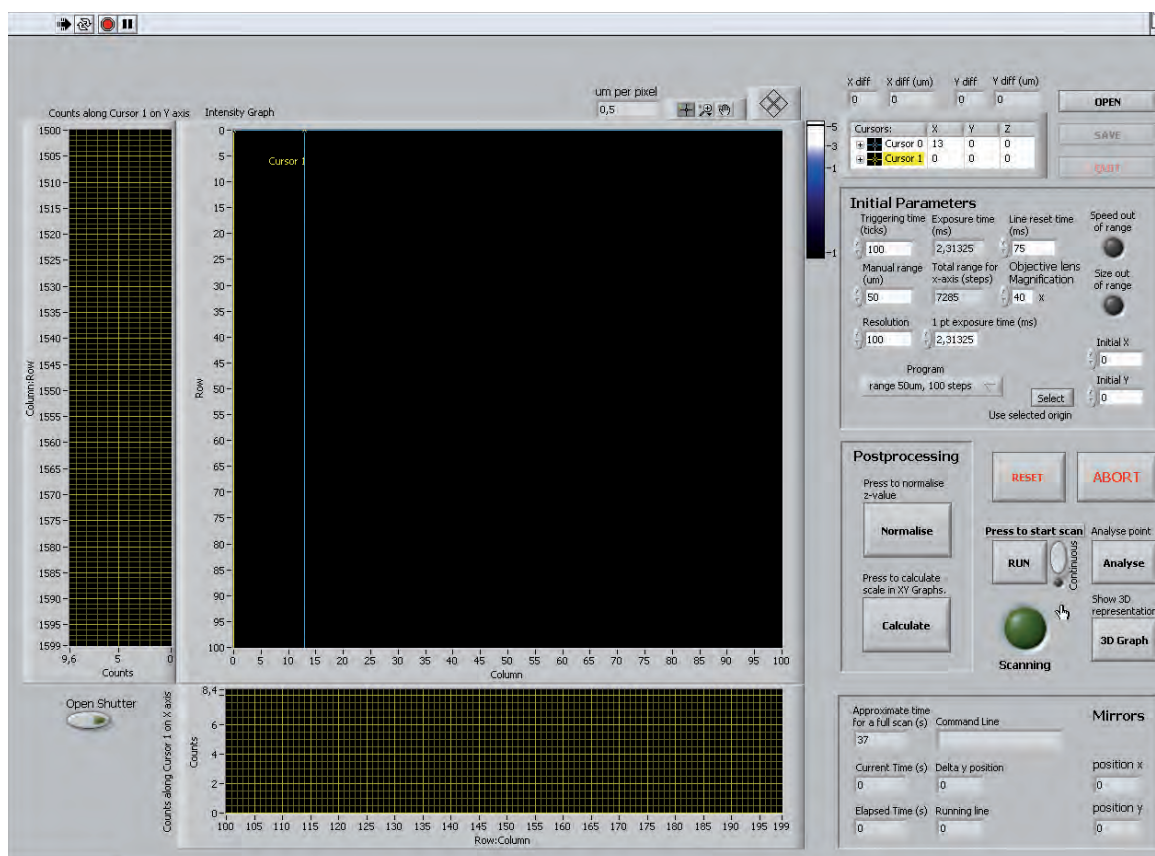


Figure 4.1 Depicting the main interface of the control and analysis software, written in LabVIEW. The system is in *Parameter entry mode* and a scan sequence will start once the **Run** or **continuous** button is pressed. Parameter fields that are user settable are white, while calculated results are grey.

The large pane is the image presented as an intensity graph in three dimensions, intensity being the third and represented as colour (note the legend on the right hand side). The panes below and to the left of it show intensity along the axes of one of the cursors.

At bottom left there is a shutter switch that can be used before starting a scan for focusing or other purposes.

At bottom right is a mirror info pane, presenting current position, line number in a scan sequence, timing estimates for scan sequences and a generated string for setting the mirrors to a certain position using the command line interface available for the mirror controller. The position displayed here is a real time readout from the mirrors showing the actual position when in *Analysis mode*.

4. Implementation – Software

The starting point for the software solution is the demonstration program suite developed by Giang Vu in his Masters thesis [1]. It successfully demonstrated how the scanning mirrors could be controlled by software and input from the sensor simultaneously recorded. However it relied heavily on nonstandard communication protocols between programs written in assembly code controlling the scanning mirrors and the LabVIEW software handling measurements, i.e. counting pulses from the APD. The synchronisation relied on sync pulses generated by the mirror controller for triggering the signal counting during the scan sweep. The sync pulses was directly relayed into the DAQ-card for the software to interpret.

This section will be largely descriptive and can to some extent even be used as a functional manual for the software. Images are included to illustrate the interfaces and certain key sections of the LabVIEW code. The full code is not included as images but can be requested from the author egh@df.lth.se or from ivan.scheblykin@chemphys.lu.se or contact the Department for Chemical Physics at Lund University (Swedish and international copyright rules apply - all rights reserved).

4.1 Basic Issues

Several systems need to interact seamlessly in order to scan the image and detect the data in a consistent manner. Apart from the controlling computer the system consists of two custom hardware instances, the data acquisition card from National Instruments mounted on the standard PCI-interface and the scanning mirrors with control hardware from GSI Lumonics which is controlled through a standard RS232 serial port.

Unfortunately the designated control computer did not carry RS232-ports, thus forcing the use of a USB to RS232 conversion dongle. This is suboptimal since the latency can be greatly increased. USB does not necessarily work synchronously, but uses buffers. The USB protocol is not strictly point to point but needs to carry addressing and such information (many devices can be connected to the same bus) [7]. In the end it would nevertheless prove possible to use the USB-RS232 scheme with little or no image distortion, depending on specified communication baud rate. Thus it was not necessary to introduce a dedicated PCI-RS232 board..

When communicating with the scan controller it is imperative that commands are delivered promptly and in a timely manner so that the position of the illuminated spot is consistent from one scan sequence to another. Further on, it was mercilessly shown that not only the properties of the physical links matter, the software's internal execution order could easily result in garbled images. An early discovery was that in an early version of the unified LabVIEW control program, the image would be right shifted by several pixels resulting in a weird folding phenomenon. This corresponded to a several milliseconds too late start of the scan sweeps as compared to the start of signal counting.

The problem could be mitigated but not eliminated by increasing the communication baud rate. The solution was in fact, as was let on earlier, making sure that the

commands that are to be sent to the controller are *logically* sent before the actual counting is started. By setting the execution order this way it is made sure that all commands have reached their destination in time.

It is wanted to easily be able to select different parameters for the scanning, such as exposure time, range and resolution. There was a very big problem accomplishing this using the solution proposed by Mr. Vu. Since the way the mirrors was controlled depended on a separate program in assembly code compiled by a special interface written in LabVIEW. This program was then to be assembled and then uploaded to the mirror controller hardware and temporarily stored in RAM (or if wanted, semi-permanently in flash memory). The specialised scan program would then need to be started at the desired time. This would be done by issuing a certain command, either using a command line interface or by using a VI directly in the LabVIEW interface, and let the counter be triggered by the sync pulses from the mirror controller. The full subsystem generation and preparation procedure was time consuming and though the system could be made to repeatedly scan a sample, it became more and more obvious that the system would be very hard to adapt to a working environment, but it nevertheless proved its point - The system is feasible.

4.2 Needed Performance

A demonstration experiment using a real MEH-PPV single molecule sample showed that for a working first scan, using a 60x objective lens with a numerical aperture, $NA=0.6$, the optical power at the sample plane was $1.45 \mu W$ (assuming a focus spot measuring $1 \mu m$, this means a power density of ca $145 W/cm^2$) and the exposure time per pixel was 2.3 ms. These parameters gave a visual result as shown in figure 4.2. It can be seen that the molecules light up significantly from the background noise.

The counter of the DAQ-board is set to count at maximum resolution as a 100×100 pixel image is scanned at the exposure time of 2.3 ms per pixel gives which gives a maximum of somewhere around **63 counts** at the peak that can be seen as the brightest spot in figure 4.2, which is a recording of the signal from this sample. 63 counts per 2.3 ms corresponds to a TTL rate from the APD of around 27000 counts per second. Assuming the APD is saturated at 5 million counts per second, only about half a percent of the full dynamic range of the APD is utilised.

The software disregards the first output value from the counter as it will be shorter than the standard length of data [1]. The control computer will thus have up to a maximum of 2.3 ms to have commands sent to the mirror controllers and have them start executing in order for a correct image to be acquired. It is also important that every line sweep is started at the same time for every line, in a consistent manner for reproducible results (do note the concept of *reset time* also addressed in section 4.4.2).

It is shown in practical experiments that it is indeed possible to produce consistent and reproducible results using the setup constructed, provided sufficient communication speed with the mirror controllers and correct internal execution order of software.

From the practical trials it was found that the background noise is about 1-5

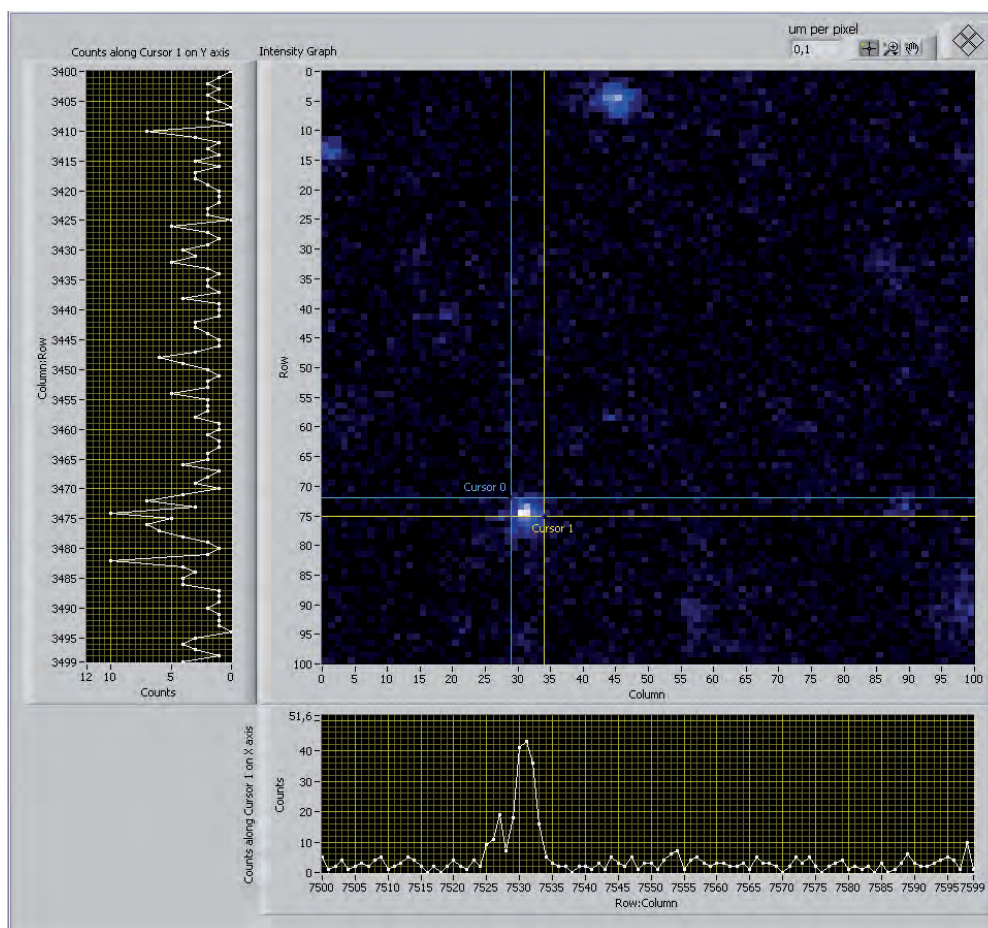


Figure 4.2 Live testing of the system. Shown are recordings of single molecules of MEH-PPV. Values along *cursor 1* is represented in the plots below and to the left of the intensity plot. The background is somewhere around 1-5 counts per 2.3 ms and the peak intensity is about 45 counts per 2.3 ms.

counts per 2.3 ms (50-250 counts per ms) and if we want to be able to see it at all we will need an exposure time of somewhere between 1 and 3 ms. This is the limit the rest of the equipment should be able to uphold.

4.3 Communication Protocols

Mr. Vu's solution did not implement any direct two-way logical communication with the controller when scanning but relied on a simple parallel interface through the DAQ-card. The controller, apart from controlling the mirrors, also carries four individually controllable "Sync" pins. These are open drain MOS-FET outputs that can be programmatically controlled. The first and second of these were connected to terminals on the DAQ-card, which was configured to read the ports as digital signals, and served to announce the state of the scan process to the counting software (the third and fourth sync pins were actually used for internal communication in the controller).

In the solution this paper describes, this kind of communication is made obsolete by introducing the unified control interface and fully LabVIEW controlled scansys-

tem. The direction of all intra-application communication was effectively reversed and changed to be logical commands sent via a RS232 serial line to the controller instead of the counting software being controlled by the state of the controller. This also means that the Master/Slave relationship between the hardware entities became more intuitive, having the computer counting the input pulses also control the mirrors directly, thus being the master. This greatly helps when e.g. there arises a desire to abort at scan session and perhaps reconfigure the microscope using other parameters, as no communication with external hardware and programming is necessary.

4.3.1 Interface Properties

A standard old fashioned 32-bit PCI card has a bus frequency of 33 MHz. The data rate supported is up to 133 MB/s. This can be compared with the RS232 serial interface connected to the mirror controller that has a data rate of at the most 57600 baud.

There is no problem measuring a data input where the *shortest count period* from the APD corresponds to a continuous frequency of up to 20 MHz, i.e corresponding to a period of **50 ns**, if duplicate count detection is used, as Mr. Vu concluded in his work [1], which should suffice as the APD saturates at a few million counts per second and the background is a few tens to a few hundreds of counts per second. It can thus be assumed that signal counting is not going to be problematic as the typical signal characteristics falls well inside the hardware specifications, as the Nyquist criterion is already included in the 20 MHz. The fastest counter on the card is clocked at 80 MHz and the duplicate count system uses this timing internally and the max repetition rate allowed is 20 MHz. Most signals in this system should normally have repetition rates below 10 MHz.

For the USB-RS232 link to the mirror controllers the situation is not as clear. The link data rate is set to 57600 baud, which was empirically shown to be the maximum possible (higher transmission rates resulted in garbled images, probably due to internal timing of the adapter or that the controller could not handle higher rates). One start bit, 8 data bits and one stop bit are used, i.e 10 bits are sent for every byte transmitted and the logical data rate is thus **5760 B/s**. This data is valid for the RS232 serial line and mirror controller.

Also, as it is necessary for the control signals to be converted from a USB interface connected to the RS-232 one it is useful too have a look at data rate magnitudes. To this data rate comparison it is worth noting the conversion between USB and RS232 incurs a latency penalty which can vary. USB operations are not always priority events. The USB host (the PC) is always initiating communication with connected devices. Thus, when a RS-232 converter is connected and even if an interrupt is used by the remote device to show that it want to initiate a transfer it is not by design so that the event is handled directly, but as soon as the host polls the converter and itself initiates a transfer.

According to USB revision 2.0 [7], such a system operates in one of several modes, such as *low speed* and *high speed*. It is noted that even the low speed mode carries data at a rate of up to 1.536 Mbit/s (192 kB/s) whereas a RS-232 interface carries at the most 230.4 kbit/s (being 28.8 kB/s). The USB interface has up to 7 times faster

response time than RS-232 and a high polling rate. The conclusion is that, as the normal scenario commands are sent to the controller over the serial interface, an action that is initiated by the host, normally, commands *should* arrive in a timely manner.

National Instruments markets a line of USB to RS-232 converters, all capable of sending and receiving at the full data rate. Intended for use with instruments e.g. controlled by LabVIEW. It can thus be concluded that the problems with garbled images when the baud rate was set too high, likely is due to the mirror controller not being capable of handling the higher data rates.

4.4 Software Structure

The basic structure consists of three logic blocks. Each of which corresponds to a state in the software. In table 4.2 the states are described. Note that due to the structure of the program, it is complicated to do arbitrary jumps between states. All transitions follow the sequence:

state 1 > state 2 > state 3 > state 1...

(See table 4.1 for an explanation of the states)

State 1 - Parameter entry

This state is the first active state. The user can input parameters such as exposure time, image resolution and physical range.

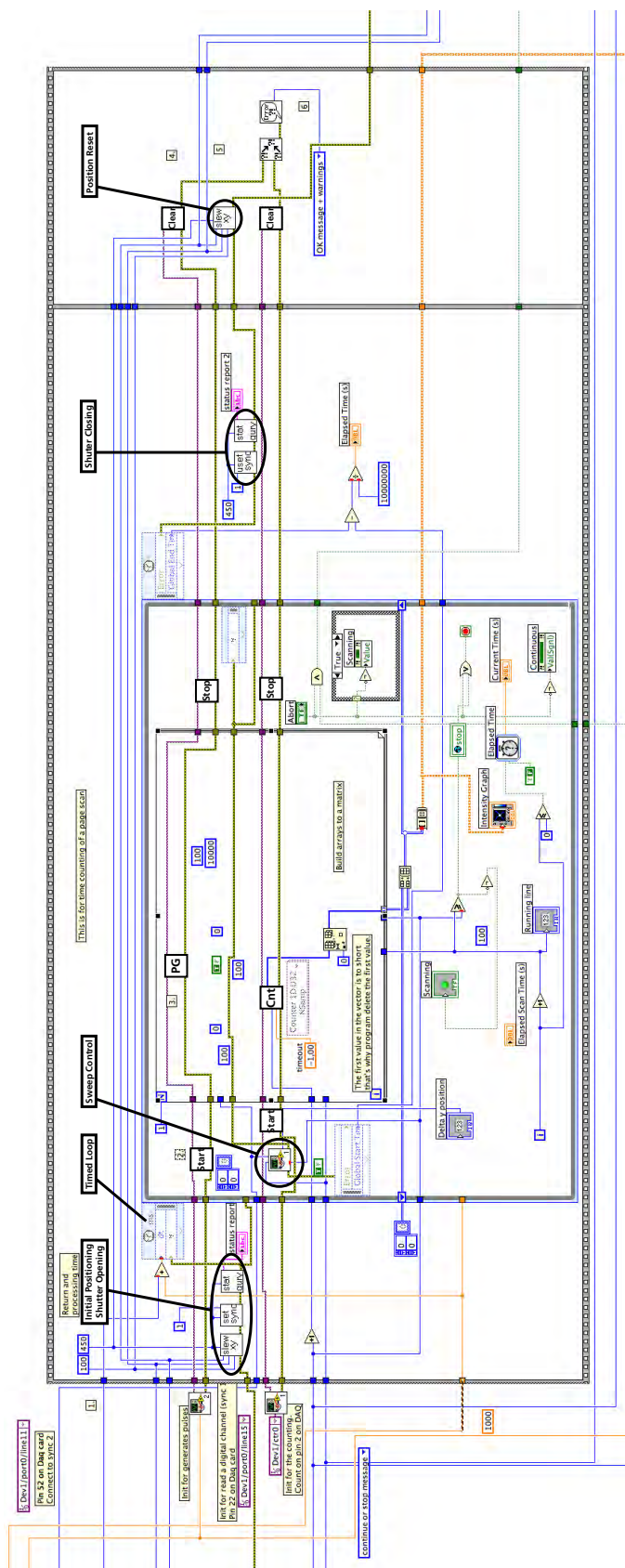
State 2 - Scanning

This state is entered when the scan button is pressed. It consists mainly of the timed loop containing the control interfaces for communicating with the mirrors and the counting subroutines working against the DAQ-card.

State 3 - Analysis mode

This state is entered when the scanning is completed. It gives the user the possibility to do a preliminary analysis of the image acquired by the microscope. Further it allows the user to select points of interest and starting a specific counting subroutine designed specifically for single point analysis.

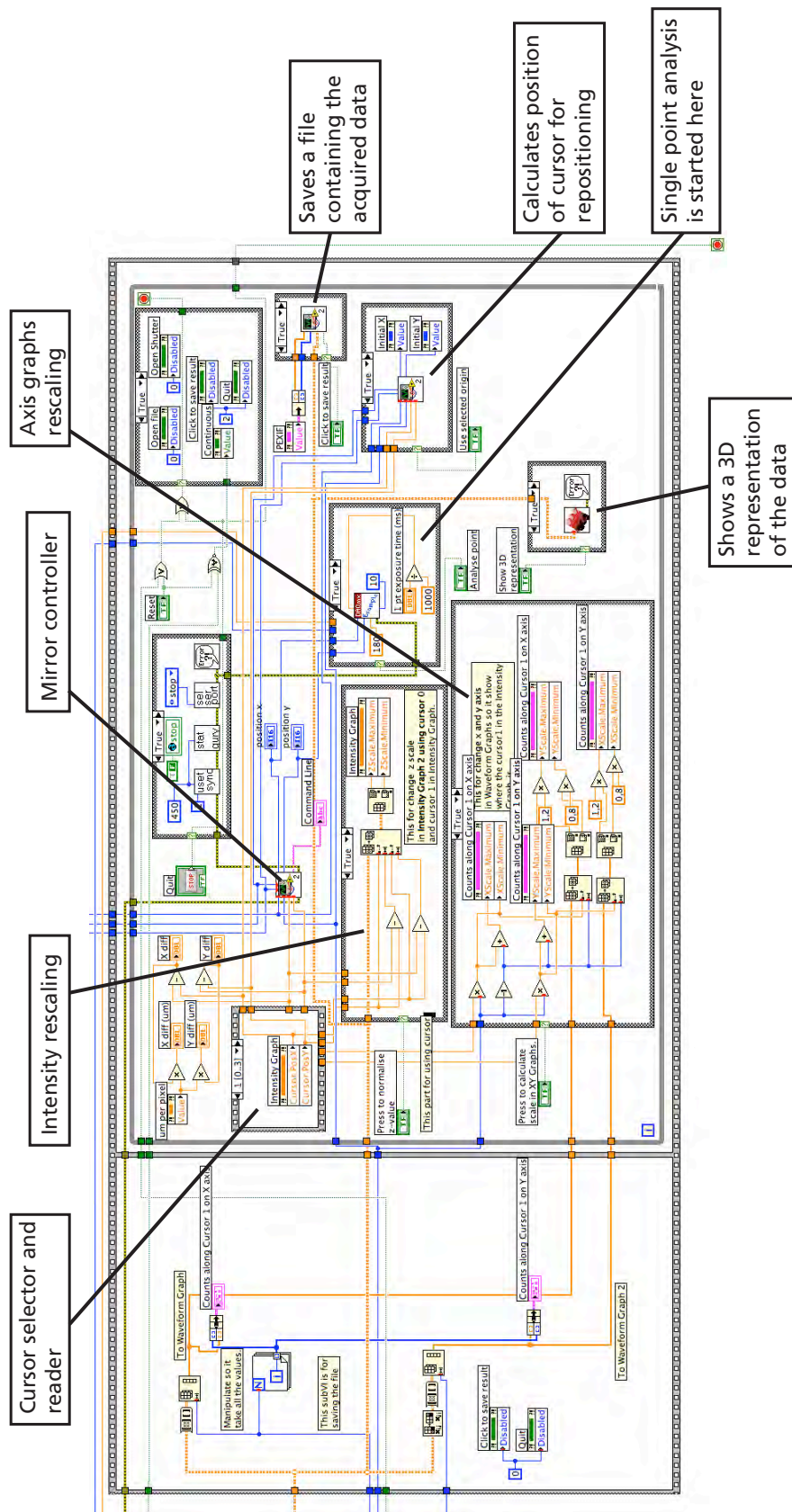
Table 4.1



Schematic 4.1 An overview of the scanning system software.

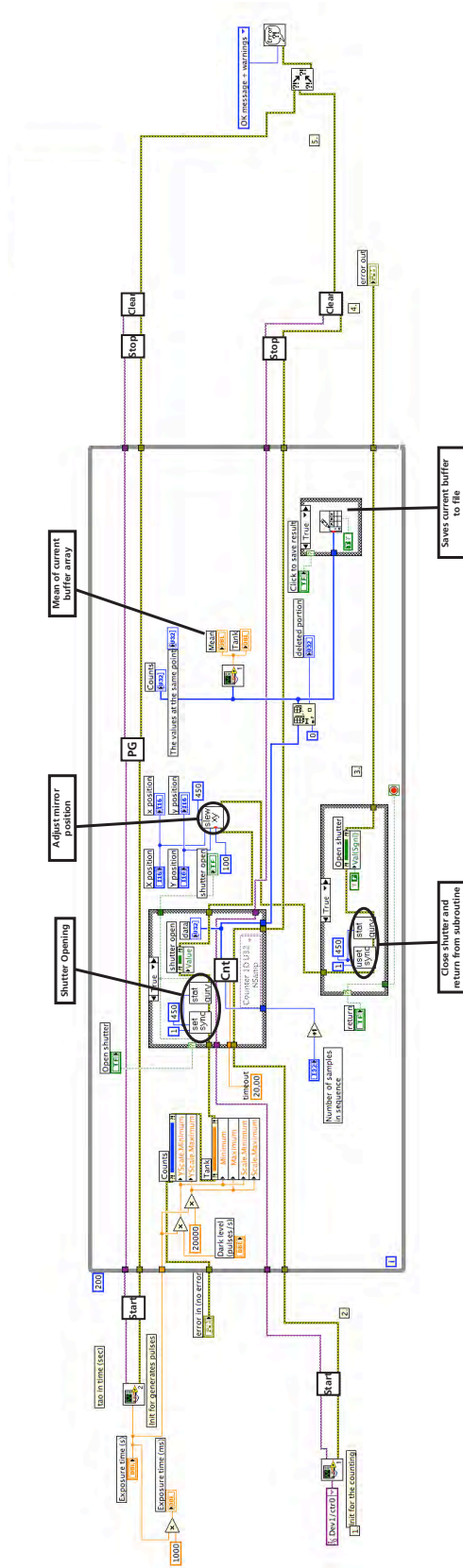
Shown here is the timed loop that triggers every line sweep, signal counting and line retrace/reset.

- **Initial Positioning / Shutter Opening** — Prepare system for scanning
 - **Timed Loop** — Start sweep at predetermined intervals
 - **Sweep Control** — Performs line sweep and retrace
 - **Init/Start/Stop/Clear** — Initialise and close DAQ card channels
 - **Cnt** — DAQ signal counter
 - **PG** — DAQ pulse generator - time base
- Outputs are the sequentially built signal matrix and several progress and status indicators.



Schematic 4.2 An overview of the analysis system software.

Shown here is the whole loop that allows for interactive control of mirrors and single point analysis initialisation. The intensity and Axis graphs are rescaled as necessary in the two sections marked. Other sections contains several practical tools, most prominently the subsystem for saving the data to file for later analysis.



Schematic 4.3 An overview of the single point analysis system software.

Shown here is the loop that repeatedly acquires a buffer array of user defined length, calculates a mean value and gives the user an opportunity to save the current buffer to file.

- **Shutter Opening** — Open shutter and start counting
- **Dynamic repositioning** — Mirror position can be adjusted from here
- **Init/Start/Stop/Clear** — Initialise and close DAQ card channels
- **Cnt** — DAQ signal counter
- **PG** — DAQ pulse generator - time base
- **Close** — Closes shutter and the subroutine and returns the user to the main interface

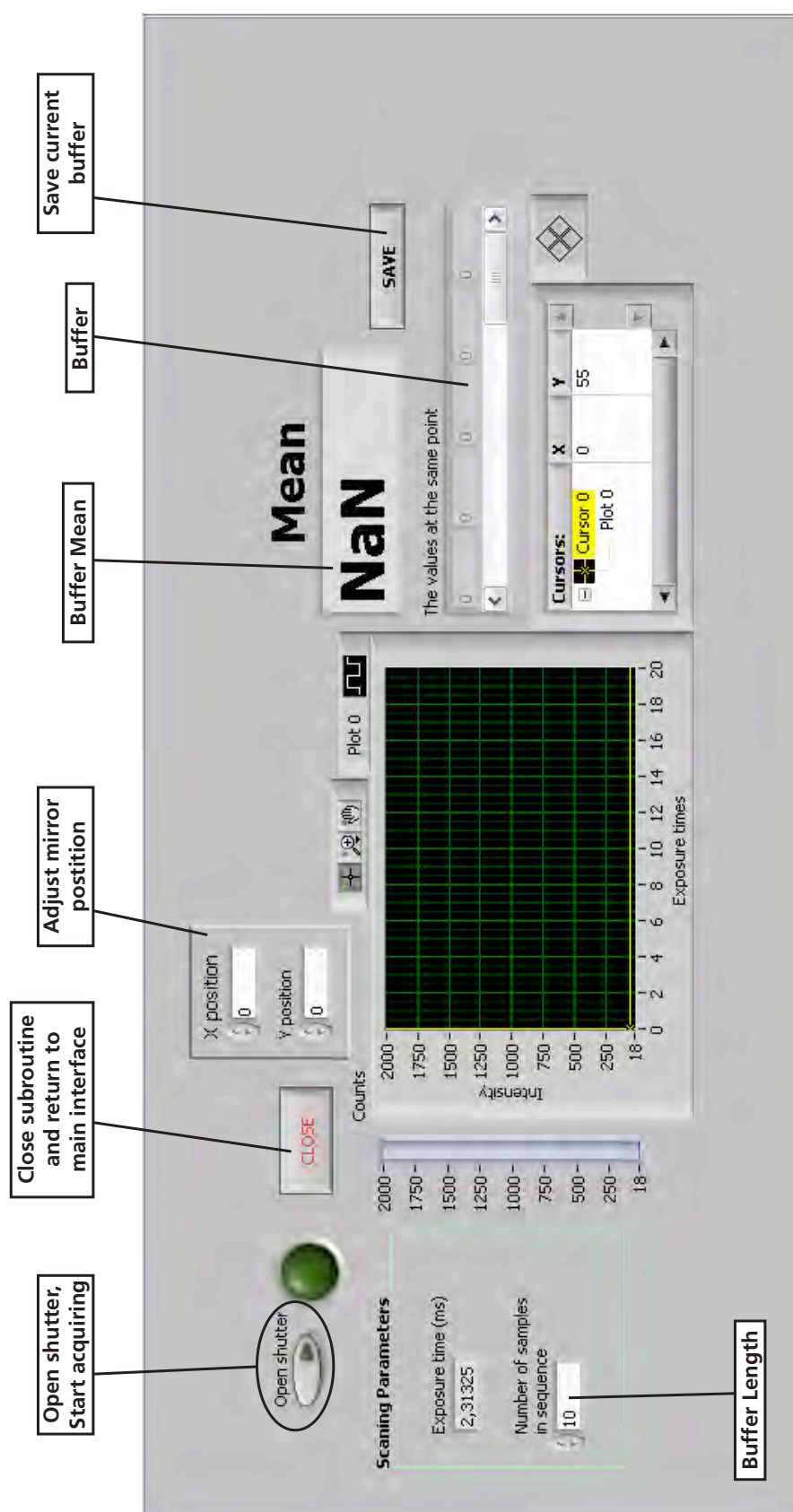


Figure 4.2 Depicting the front panel of the single point analysis system software.

There exists an abort function which interrupts the scanning while in progress and gives a shortcut to the analysis state, It is well worth noting that the analysis state is not fully functional after an abortion since it assumes that a full scan has been completed. The recommended way to proceed is to do a reset and begin again, *or* if a suitable molecule is discovered directly start single point analysis (the use of this method is deprecated since for its use it is vital to keep in mind the absolute coordinates for the location of the molecule since it is not certain that positioning using the cursors will work as expected if a scan sequence is aborted prematurely).

The *reset* function restores the system from the analysis mode to the first, parameter entry state. Thus a reset is always needed before the next scan cycle can be performed even if the user wishes to use the same inparameters.

4.4.1 Software Block 1 - State 1 - Parameter Entry

The second and third software blocks are based on the BufferedCounter VI developed by Giang Vu. The first block was originally just a set of standardized inputs that the user had to set once before starting the program. The need to dynamically change the parameters became obvious when the program gradually expanded as the list of parameters needed for correct operation and userfriendliness grew. Inputs for physical parameters of the microscope such as objective lens magnification, numerical aperture and starting point allows the user to get correct sizes in the image recalculated to real-world units presented by setting the built in measurement cursors, and to set a new starting point e.g. if an interesting area is somewhere near the end of the scan cycle it is possible to start scanning close to the interesting area. This feature also provides zooming, i.e. choosing a new starting point and changing the scan parameters sweep length and resolution. This *will* result in changed exposure times and radiation doses per molecule unless compensated for. This is *not* done automatically in the present software.

Other useful features are the preset ranges giving the user the possibility to select standard ranges such as maximum range (as dictated by the mirrors physical parameters), a standard range where one pixel approximately corresponds to the resolution limit according to the Rayleigh criterion (for further discussion of this, see section 2.2-2.4), and finally a fully manual mode. Several different pre-programmed resolutions are also available and more may easily be added by someone with some minor experience in LabVIEW programming.

4.4.2 Software Block 2 - State 2 - Scanning

The second block is as mentioned before based on Mr. Vu's scanning counter. Some major refactorings can be found though. The content of the basic structures controlling the DAQ-card can still be found and have not been altered significantly, whereas the structures around these parts have changed. Since the refactoring to control the mirrors from within the LabVIEW program the loop for counting detected pulses for each line is not controlled from the outside via a signal from the mirror controller but is superseded by a timed loop making sure that every line is scanned in a timely manner.

Provisions are given in the first block to manually set the *reset time* needed for

mirror retracing. The way retracing is done has been changed from the method Vu used. Instead of first retracing along same axis as the scan and then repositioning for the next line, a zig-zag pattern is used (see figure 4.3). This should further reduce the dose minutely. What turned out to be a good standard value for retracing is ca 75 ms. This is one of the optimisation parameters and it needs to be sufficiently long for the mirrors to reset for the next sweep and stabilise (any oscillations induced by a quick retrace should have time to die out), but preferably not too long as it multiplies with the number of lines and adds to the total time needed for a full scan.

This is where the biggest challenge turned out to be; in order to be able to capture an image in a deterministic fashion it was necessary to make sure that the actual line sweep and the line pulse counting started, if not exactly at the same time, at least the physical sweep must have started before the line counting starts and everything must

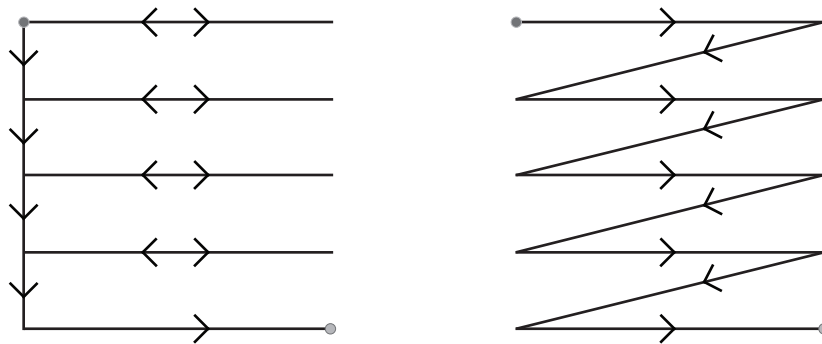


Figure 4.3 Scanning methods for Giang Vu's solution (left) and the solution presented in this paper (right). Starting points at upper left corners are marked in light grey and end points at lower right corners are marked in darker grey.

happen at the same time of the sweep every time. This was actually a major problem until a redesign of the call order inside the timed loop was done in order to fulfill the necessary conditions.

If the system suffers bad synchronisation the problem will reveal itself as a vertical stripe of noise at the beginning (left hand side) of the captured image (it is possible that a similar effect can be provoked to occur on the right hand side of the image as well, depending on the set properties of the timed loop, but at least with the current program it should be unlikely or even impossible to do this accidentally). Also, it was necessary to use some sort of calibration sample to make sure the images were consistent. If more than two consecutive scans give the same positions of all points on a calibration target it can reasonably be assumed that the image is true to reality. This consistency is crucial for the system to be usable. Some compensation can be made for discrepancies between the scanning mode and the single point analysis mode as long as the error is known and deterministic. Fluorescent beads, a few μm in diameter, were used as standard objects to ensure image consistency between scans. See figures 5.1a and b for such images.

The structure of the scanning subsystem is depicted in **schematic 4.1**. The timed loop contains the counter and line sweep controller and shift registers are used to build an image matrix that is presented in the intensity graph. Other features that can be seen are indicators, a timer for total elapsed time and the shutter controls, using the sync pulses available on the DAQ-card.

4.4.3 Software Block 3 - State 3 - Image Analysis, Aiming and Single Point Analysis (Live capture of single points)

This block is the part of the program that bears the most similarities to Mr. Vu's proposal. The way data is collected from the APD-DAQ chain and the image matrix is built in block two are the same. The way timing is handled is different though. The third block is entered after the matrix has been filled (or the abort button has been pressed). It has two separate analysis VI:s whereof the primary one is a part of the main program and the second one is mostly identical but is placed in a separate pop-up VI and analyses a previously saved data file, it is started when the user wants to open a saved data file.

There is also a simplified counter in a separate VI that works like the one in the scanning block, but instead of sequentially building a matrix from the values it presents a vector with a size that can freely be chosen before the block is entered (i.e. the *Analyse Selected Point* is pressed). This counter is based on mr. Vu's *BufferedCounter at 1 point* but just like the main scanning system, the handling of timing is rewritten. Also, there are provisions for controlling a shutter via the sync channels on the controller here as well. In this VI the shutter button also acts as a start/stop button for the counting. The system is constructed so that after the shutter button is pressed (its value set to *True*) it will sequentially record vectors of chosen length, though only retaining the most recently recorded which will remain in memory after the button has been unset. After unsetting the data collection will continue until the current vector is filled whereafter the shutter is closed and the user is given the possibility to save the vector to file. This functionality is intended to be versatile and useful for measurements on e.g. fluorescence lifetimes. Also added is the possibility to control the mirrors, letting the user move a few steps while working with this VI (or, if wanted, even longer distances), useful if one wants to investigate several points during one session (the interesting positions then needs to be known beforehand). It shall be noted that the way the system works, after pressing the shutter button the counter fills the buffer and then it starts to refill it. The second pressing will still fill up the current buffer (vector) before closing the shutter. The data is then retained until the window is closed or the shutter button is pressed anew. A *save buffer* functionality is included. Saving the content is done to a spreadsheet file which can be imported to MATLAB or whatever analysis software is preferred.

The image analysis VI:s (the main VI and the file analysis VI) have two cursors that is used for normalising the brightness of a recorded image. Using these the user draws a square around the area that contains interesting data and presses *Normalise* in

the *Postprocessing* pane and the software adjusts the brightness of the image. There is also the *Calculate* button which gives a representation on the graphs along the axes the brightness along one of the cursors, designated *cursor 1*. This is shown in figures 4.1 and 4.2. The same cursor is connected to the mirror control and with its hair cross it is possible to select a point on the sample to illuminate, e.g. for single point analysis or intentional photobleaching. This way you can also select a new starting point on the sample for a new scan. If the user wants to do this, the position can be loaded as a new starting point by pressing the *Use selected origin* button in the *Initial Parameters* pane. The structure of the cursor functionality and the general analysis software layout is depicted in **schematic 4.2**.

4.5 File Formats

Data retention is always needed in a system intended for scientific work so naturally one of the most important aspects of the system is how it saves collected data to files. Normally two different situations occur during normal operation: *Images*, overviews of sample the sample area are acquired, mainly for locating molecules but the data can be used just as easily as in the second case: *single point measurements*, in which case time series can be recorded. For these different situations two different data file formats are used and one support file format, the Pseudo EXIF file format (file ending .pexif) containing metadata concerning the image files.

The two main file formats is a binary bitstream type, used for initial image data retention. This can then be converted into a text file format. The image is in that case represented as a text matrix of the values. The same type of file is used in the single point analysis pane, a text file containing an array of the acquired values is saved. This format can then easily be imported into e.g. MATLAB or Excel for further processing.

4.5.1 Image Files

Image data can be saved to file after scanning the sample, using the button *Save* at the top right (see figure 4.1). The file format is a binary bitstream pattern and needs to be read by the file analysis pane using the *Open* button. The file format is appended an file ending *.spe* that has been chosen by chance but it should not interfere with most other applications (the file analysis application assumes this file ending, but any ending should be viable). Since LabVIEW is very easily changed with respect to file naming conventions, this can be changed according to taste at any point. The files are read in and the image size is determined by the file length and it is *implicitly assumed* that the image is *square with equal sides*. If the data is needed outside the system there is a way to convert the data into a spreadsheet format (pure text file) on the file analysis pane. The convention is that whitespace separate values (columns) on a row and newline separate the rows.

An external file analysis tool without interconnection to the scanning system has been constructed for pure conversion and analysis tasks that can be run on any system with LabVIEW installed. This conversion tool lacks the 3D representation tool that is

included in the main software. The 3D tool relies on software subroutines specific to the *Microsoft Windows* operating system (DirectX). By excluding this part the software can be used on other systems such as Mac OS X (tested) and Linux (untested).

4.5.2 Single Point Files

Time series can be saved using the single point analysis tool. The counts acquired during a measurement are binned according to a preset time base and the last acquired data array can be saved to a text file similar to the secondary image file format described above, newline separate the values (i.e. one column with one value per row).

4.5.3 The Pseudo EXIF File

Images saved to the binary file format as well as the text file format does not carry any metadata, in this case being information on what ranges were used, what objective lens, point of origin and so on. Thus the *Pseudo EXIF (PEXIF)* file format was invented. The name is chosen to be reminiscent of the EXIF metadata field found in e.g. JPEG and other image file formats. As the metadata is not saved into the actual data file, it is saved to a separate text file called PEXIF (file ending *.pexif*). It is not even desirable to insert it into the data files due to different reasons: for the bitstream file the size of the image is determined from its file length which would be altered by inserting extra data and for the text file the target program is not known and comment markers are not known beforehand (e.g. MATLAB uses '%' while other software might use other characters like '#' or it may even not have such an escape character). It is true that some file system tricks could have been used to put the data in some metadata field there (as e.g. putting it in a *Resource Fork* in HFS or HFS+), but it is not certain that this information would be cross platform readable. It is also perhaps more intuitive to have an external file with the same file name except for the file ending appearing alongside the data file.

The content of a typical PEXIF-file is shown in figure 4.4. Some of the data in the file are values the user will have to enter in the main scan pane, such as magnification of the objective lens and point of origin of the mirrors. These values allow the user to later recollect the correct scales and sizes of objects found in the image.

```
This is a Pseudo-EXIF file for: \\athena\CHEMICAL PHYSICS\Confocal\Saved Data\
Mirror2-burntcenter-20090115.spe

Saved 2009-01-15 18:26:39
-----
Objective lens used: 40x

Exposure time: 1,156625ms corresponding to 50 ticks of 23us
Resolution: 100x100 pixels
Range:      50,00um corresponding to 7285 steps
Steps:      73 steps per pixel, corresponding to 0,5000um per pixel

Starting position:
X = 3000
Y = -3500
-----
```

Figure 4.4 Pseudo-EXIF file. Metadata for a saved scanned image.

5. Results and Conclusions

During this project it has been shown that a confocal microscope, built around mostly standard components can have enough sensitivity to be able to resolve and perform spectroscopic analysis on singular molecules on a planar surface. Experiments has shown that detection and imaging of positions of a polymer such as MEH-PPV spincoated or smeared on a glass surface can be done using the microscope. The molecule density on the carrier needs to be less than 1 molecule per square micron in order to properly resolve the individual molecules. Slightly better resolution is theoretically possible, but given the requirements, this spatial resolution is acceptable.

It is clear that a confocal microscope built like this will need to be recalibrated frequently, as no setup built on an optical bench is perfectly stationary. At least once a week the setup needed to be fine-tuned as the beams did no longer pass correctly through the detector line.

Focusing is a complicated procedure and needs to be done with the live sample carrier to find the correct plane. The reflection method described in section 3.3 proved to be the only working method due to the same reasons as the need for calibration. No two samples are perfectly adapted for the system and the system is not a static entity. This also means that the sample will be exposed to stray light during focusing, which may take some time, but nevertheless it should still be exposed to a smaller *dose* than would be the case in a widefield microscope counterpart.

5.1 Sensitivity

The system was able to exhibit a maximum signal to noise ratio of roughly 12:1 when imaging MEH-PPV. In decibels this is 10.8 dB. This is quite low when compared to the widefield system used at Chemical Physics and should be fully possible to improve. The advantage the confocal scheme has when it comes to resolve intensity from the focal point and suppress noise from outside it (equation (5)) should cater for a very good SNR for a well tuned confocal microscope.

It should also be noted that the minimum beam waist of the at the objective lens focus is very hard to obtain. A reasonable assumption is that the real size of the illuminated spot is up to ten times the theoretical minimum, so the power density calculations in section 2.6 should be read with this in mind, as the power density scales with the actual size.

5.2 Software

The software was originally built around an imaging solution. This idea persists to this day, but the usability has greatly improved when compared to the demonstration software written by Giang Vu. The reworking of the whole hardware control interface and its conversion into 100% LabVIEW code greatly improved the opportunities to do further work on the code at the same time it lets users quickly set parameters and get going. Another advantage is that the logic is centered to the control computer and the number of physical as well as logical channels to the controller is minimised to the serial line

(the communication through the DAQ-card is no longer needed). As no logic is located in the controller anymore, execution order internally in the LabVIEW programming became much more crucial and quite a few timing loops needed to be rewritten before correct and consistent images could be acquired.

The imaging solution, uses intensity graphs with either linear or logarithmic representation in the z-axis (intensity axis). The representation can be chosen to what suits the situation best. In addition to this standard imaging solution the user can chose to view the intensity in a 3D representation. This tool can prove useful when searching for weak signals with high background noise. See figure 5.5-5.6. This software module uses libraries that are Microsoft Windows specific (DirectX) so it will need to be removed or in another way disabled if one wants to convert the software for use on a different platform.

5.3 Images

Some interesting images were acquired during the course of the project. First of all, in order to verify the microscope's imaging capabilities and the accuracy of the built in scale conversions preparations were made with fluorescent beads with had an consistent size. When it could be shown that several consecutive scans of an area that was supposed to be the same, gave the same image for each and every scan it was proven that it actually were the same area being imaged. When this was proven using these beads, single molecule samples could be tried. In figure 5.1 such bead images are shown. Another image used for calibration is the one shown in figure 5.2, it is a depiction of a 30 μm pinhole.

In figure 5.3a is shown an image taken of a single molecule sample as taken by the widefield microscope setup at the SMS group. It shows single molecules fluorescing as glowing points against a dark background. A similar picture, taken with the confocal microscope is shown in figure 5.3b. The molecules show up as bright spots. Every spot is an image of the PSF and corresponds to a spot about 1 μm diameter.

In figure 5.4 the blinking effect due to quenching effects is apparent. In this case the lifetime of the dark state cannot be established with absolute certainty as the laser is sweeping so if the period just happens to time the passage of the laser it might be a shorter lifetime but it is obvious that the apparent lifetime is three and a half rows of the spot. Every pixel corresponds to 2.3 ms, each row consists of 100 pixels, ergo 230 ms for a row sweep. Finally there is the reset time for retracing of 75 ms. So the apparent lifetime of the dark state is ca 1 s.

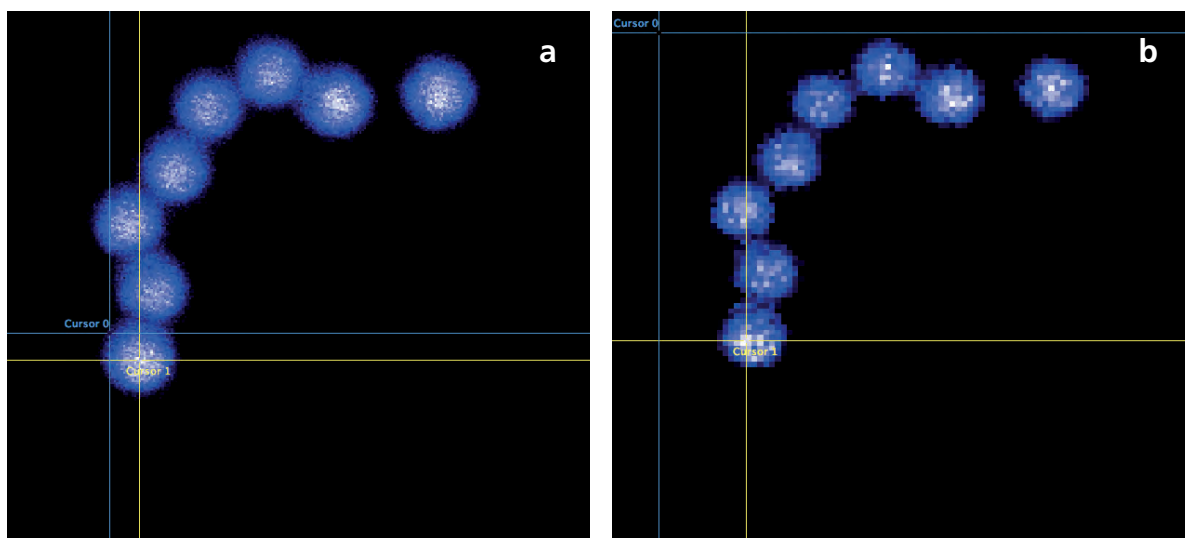


Figure 5.1 - A series of fluorescent beads, about 10 μ m diameter, used for instrument calibration depicted at different resolution but the same zoom rate. The left image, a, is recorded at 256 points per row, the right, b, at only 100 points per row.

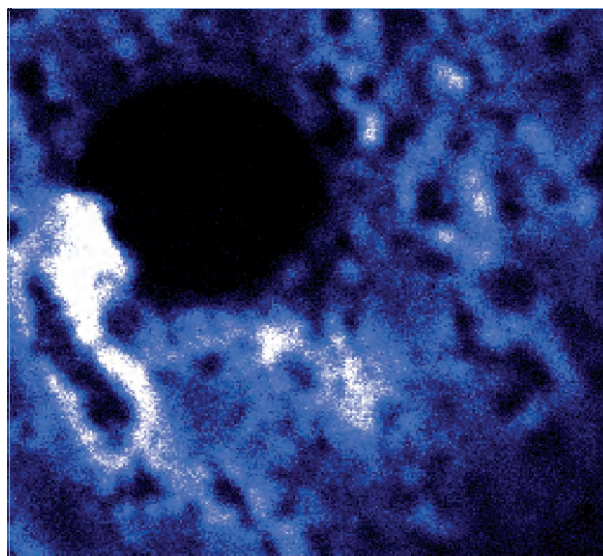


Figure 5.2 - A 30 μ m pinhole placed in the sample plane and imaged by the microscope during calibration runs.

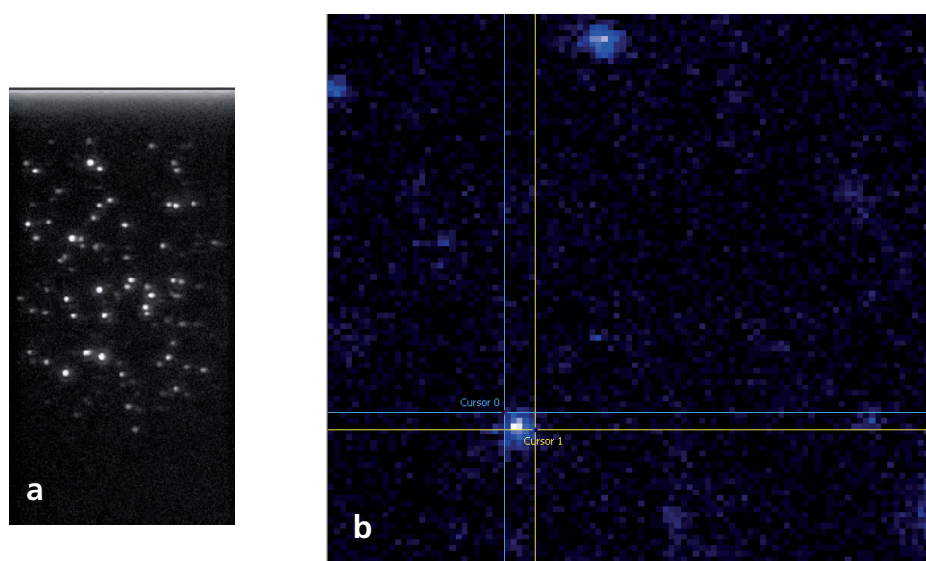


Figure 5.3 - Samples of MEH-PPV viewed through a widefield fluorescence microscope to the left and through the confocal microscope to the right

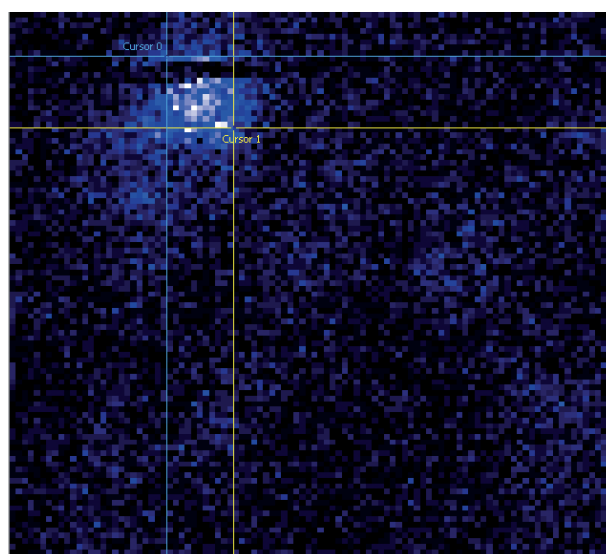


Figure 5.4 - Sample of MEH-PPV exhibiting blinking due to quenching

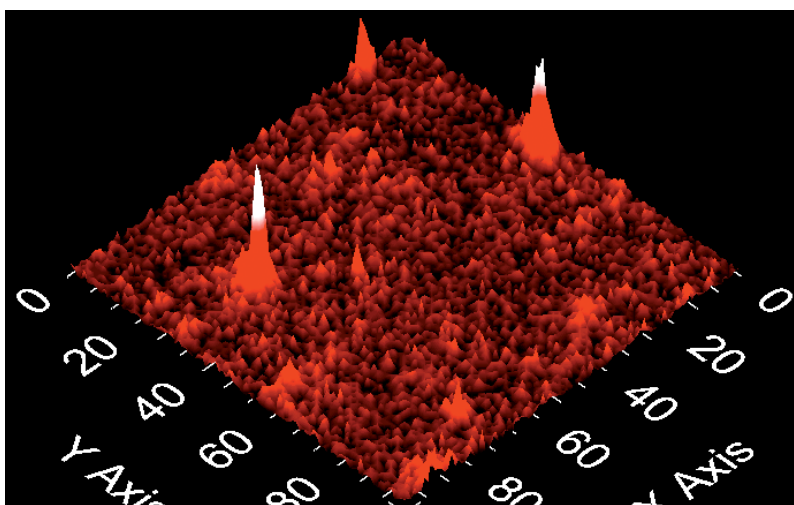


Figure 5.5 - 3D representation of the same data as in figure 5.3b

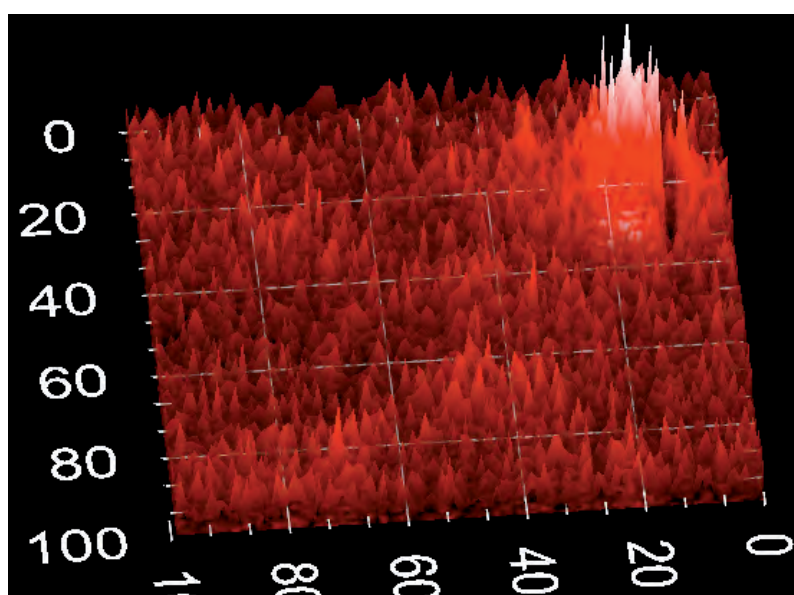


Figure 5.6 - 3D representation of the blinking MEH-PPV. Same as in figure 5.4

6. Further Development

There are some improvements that can easily be added to the system to further increase the systems capabilities. Some of them being actual rebuilding of the optical setup and additions to it and other are improvements of the software. It all depends on the users intended use pattern.

6.1 Hardware

Several ways of adding functionality to the existing setup are imaginable. A spectrometer can be added at two different places in the setup. One is to place a (preferably flippable) beamsplitter in the parallel beampath between the objective and the tube lenses. A spectrometer placed here can use the reflected laser excitation light as a reference with proper filtering. The second possible placement is in the detector line also using a beamsplitter or, even better, a flippable mirror.

One approach that has not been covered in detail in the current setup is the possibility of using two photon processes in the analysis. This would require a reworking of the beampath around the dichroic mirror and the focusing will be more difficult, but a proposal for alternative beam plans are shown in figure 6.1-6.2.

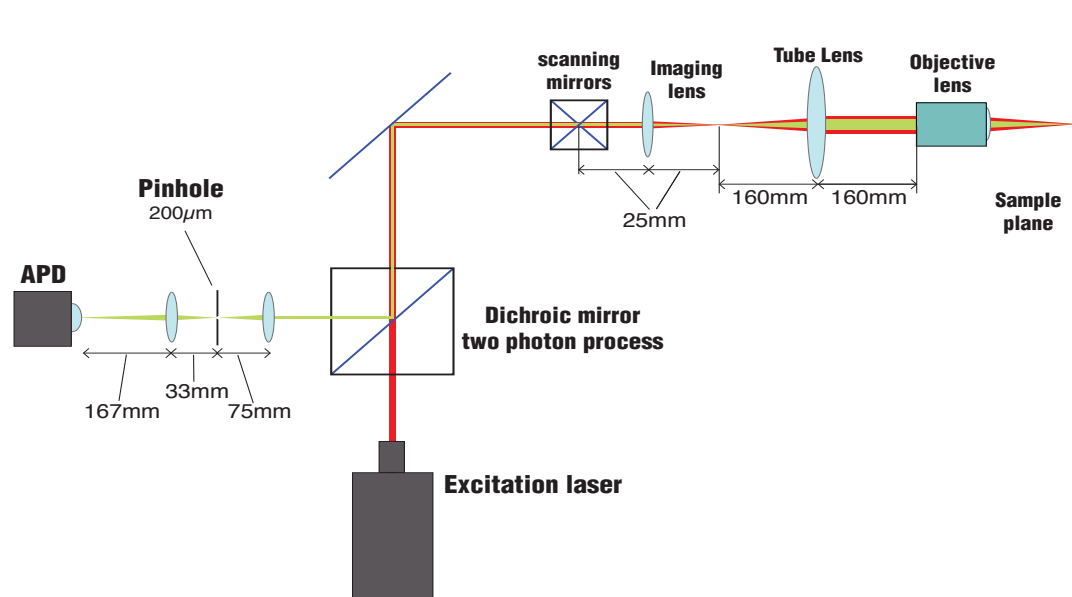


Image 6.1 Setup proposal for scanning methods using two-photon process. The convolution has changed in this case the excitation light passes through the dichroic and the fluorescence is reflected

The more complex version is a way of rearranging the system for use both with one and two-photon processes without rearranging the detector line orientation. Using flipping mirrors or even beamsplitters, if aligned correctly (regular recalibration on something like a weekly basis will most likely be necessary) the single-photon line can be used for

focusing and primary scanning and the two-photon line for e.g. single point analysis.

It is easy to introduce polarisers at several points of the setup. The best choices are any of the path sections with parallel light.

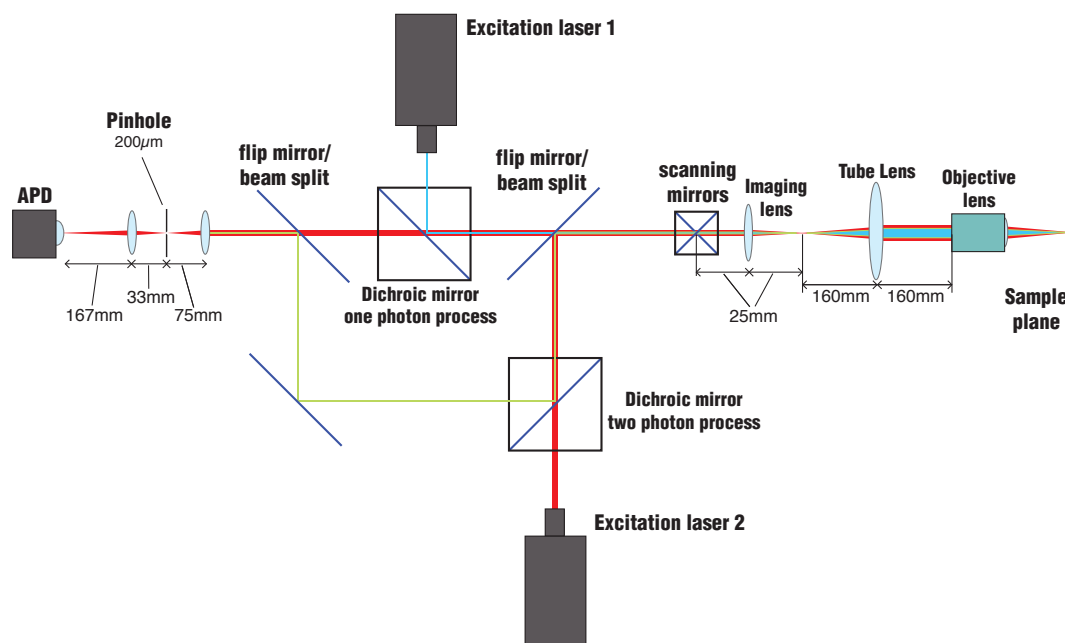


Image 6.2 Setup proposal for a combined system using both two-photon and single photon processes in the same setup. Flippers or beam splitters can be used.

To increase the spectral selectivity a chain of dichroics and multiple sensors can be added. Another option is the use of a grating or even a standardised spectrometer, provided that the quantum yield is high enough to give a significant response.

6.2 Software

There are several points that can be addressed in order to make the software more user friendly. A few strange units are used in the input parameters due to the design of the mirror control hardware. The most obvious one is the fact that exposure time is still denoted in hardware specific ticks of ca 23 μ s. It can be desirable to just input the exposure time. A dosage calculator into which, desired max dose, laser beam power and wavelength is put in can be a future addition.

Also the scanning range is reported in number of steps of the galvanometers, even though the input is in μ m. In a more mature system this might not be necessary to show by default, but at present these numbers can come in handy at times.

A focusing aid using either the APD or a separate CCD camera integrated into the interface could very well prove very useful since no secondary applications are needed and the single point analyser does not need to be used for this.

Appendix

A.1 Microscope Imaging

The following formulæ was found relevant for the theoretic description of microscopes in general and confocal microscopes in particular. Referenced works are Webb [2], Wilson [3] Siegler [15] and Smith [20]. The nomenclature in this appendix is mainly Webb's.

A.1.1 Contrast

To explicitly address background/stray light (noise) the following extension to (7) can be made. First the intensities n_b and n_d is divided into signal and background like so: $n_b = b + B$ and $n_d = d + B$. This means the contrast (equation (7)) can be rewritten:

$$C = \frac{2J_1^2(\rho)}{\rho^2} \quad (14)$$

The signal and noise relations are:

$$S = n_b - n_d \quad (15)$$

$$N = \sqrt[4]{n_b^2 + n_d^2 + 2n_{\text{stray}}^2} \quad (16)$$

And the signal to noise ratio (SNR) will be:

$$\text{SNR} = \frac{0.84(n_b - n_d)}{\sqrt{n_{\text{stray}}}} \quad (17)$$

Where n_{stray} is stray light intensity. This shows the influence of stray light on the system.

A.1.2 The Point Spread Function

The point spread function, $p(\xi, \rho)$ in the focal plane, $\xi = 0$, is written:

$$p(0, \rho) = \frac{2J_1^2(\rho)}{\rho^2} \quad (18)$$

J_1 is the first order Bessel function:

$$J_1(\rho) = \frac{1}{2\pi} \int_0^\pi e^{-i(\tau - \rho \sin \tau)} d\tau \quad (20)$$

Since a confocal system where the same lens is used to convey the excitation light to the sample and to image the fluorescence, mathematically can be described as two identical optical systems aimed at the same sample the total psf will be the product:

$$p_{\text{conf}}(\zeta, \rho) = p_{\text{exc}}(\zeta, \rho) \cdot p_{\text{img}}(\zeta, \rho) = p(\zeta, \rho)^2 \quad (19)$$

A.2 Absorption Cross Section

An atom or a molecule physically has a diameter much smaller than the wavelengths used in optical microscopes and as such will not show as a discernible shape when imaged. Neither will they absorb light arbitrarily when illuminated. A molecule will appear to have a certain *cross section*, σ , that is wavelength dependant which determines how it absorbs energy.

When illuminated by the power density $I = P / A$ the power absorbed, ΔP_{abs} , by a specific molecule is [15]:

$$\Delta P_{\text{abs}} = \sigma \cdot (P/A) = \sigma I \quad (21)$$

B. Hardware List

The instrument was built with standard components. The following is a list of main components (not including all nonspecialised mounts and such):

Olympus Microscope – divided into sections

Ocular/Eyepiece - used as imaging lens, mounted on xyz-stage

Tube lens - modified fitting, mounted on adjustable lens mount on xyz-stage

Microscope stand - objective lens mount and focusing

Scanning Mirrors

GSI Group Corporation scanhead,
6 mm VM500+

Scan controller

GSI Lumonics scan controller, SC 2000



Image B.1 Scan controller and microscope stand

Dichroic Mirror

Olympus filter cubes - UIS2 series [10], slight modification, cleanup filters removed from package and mounted later in the detector line

Collecting optics

Pinhole collecting lens, focal length 75 mm

APD collecting lens, focal length 100 mm

Pinhole

Interchangeable - normal aperture values, 100 μm or **200 μm**

Avalanche Photo Diode (APD)

Excitation light source

Laser: wavelength, CW or pulsed, depending on experiment

Control computer

Standard PC - software requirements: LabVIEW v8.4 or later, GSI Lumonics drivers installed

Data acquisition card (DAQ)

National Instruments: NI 6602

Several custom mounts

Built as needed

Camera Obscura

A custom made shadow cabinet to shield the APD from stray light, doubles as a nd-filter holder. Built to suit intended APD housing.

Bibliography

- [1] Giang Vu
Software Control of a Confocal Microscope
Department of Measurement Technology and
Industrial Electrical Engineering — Lund University
(Department of Chemical Physics — Lund University)
(2008)

- [2] Robert H Webb
Confocal optical microscopy
Schepens Eye Research Institute and Wellman Laboratories of Photomedicine,
Massachusetts General Hospital, Boston, MA 02114, USA
IOP Publishing Ltd. (1996)

- [3] T. Wilson
Confocal Microscopy
Department of Engineering Science
University of Oxford, UK
Academic Press Limited (1990) pp 1-64

- [4] Oleg Mirzov
Single-molecule spectroscopy of π -conjugated polymers
Department of Chemical Physics — Lund University
(April 2008)

- [5] Daniel Thomson
*Single Molecule Fluorescence Spectroscopy of π -Conjugated Polymers:
Polarization Fluctuations and Electric Field Effects on Intensity*
Department of Physics — Lund University
(September 2006)

- [6] OSHA Cicinatti Laboratory
Cincinnati Technical Centre.
Electromagnetic Radiation and How It Affects Your Instruments.
“Near field vs. Far field.”
(May 20, 1990)

- [7] USB Specification
Universal Serial Bus specification, Revision 2.0
(April 27, 2000)

- [8] Sune Svanberg
Compendium on Multispectral Imaging
Department of Physics — Lund University
(2005)

- [9] Sune Svanberg
Atomic and Molecular Spectroscopy
Springer Verlag
Fourth Edition (2004)

- [10] http://microscope.olympus-global.com/uis2/en/fl_mirror/
Olympus UIS2 Series for Biological Microscopes
Dichroic filter cube specifications
(collected 23rd November 2010)

- [11] W. E. Moerner, David P. Fromm
Methods of single-molecule fluorescence spectroscopy and microscopy
Department of Chemistry, Stanford University
Review of Scientific Instruments, volume 74, number 8, August 2003
pp 3597-3619

- [12] Xavier Michalet, Shimon Weiss
Single-molecule spectroscopy and microscopy
Department of Chemistry and Biochemistry, UCLA
C. R. Physique 3 (2002) pp 619-644

- [13] Q-T. Nguyen, N. Callamaras, C. Hsieh, I. Parker
Construction of a two-photon microscope for video-rate Ca^{2+} imaging
Laboratory of cellular and Molecular Neurobiology,
Department of Neurobiology and Behavior, University of California, Irvine
Cell Calcium (2001) 30 (6), pp 383-393

- [14] N. Callamaras, I. Parker
Construction of a confocal microscope for real-time x-y and x-z imaging
Laboratory of cellular and Molecular Neurobiology,
Department of Neurobiology and Behavior, University of California, Irvine
Cell Calcium (1999) 26 (6), pp 271-279

- [15] Anthony E. Siegman
LASERS
University Science Books
(1986), pp 286-291
- [16] Nathan S. Claxton, Thomas J. Fellers, Michael W. Davidson
LASER SCANNING CONFOCAL MICROSCOPY
Department of Optical Microscopy and Digital Imaging, National High
Magnetic Field Laboratory, The Florida State University, Tallahassee
- [17] Tony Wilson, Martin Friedrich, Alberto Diaspro (editors)
Basics of Light Microscopy & Imaging
GIT VERLAG GmbH & Co. KG
- [18] Olympus Microscope
Olympus UIS2 Microscope Components Guide
(2006-11)
- [19] Joseph R. Lakowicz
Principles of Fluorescence Spectroscopy
Springer Verlag
Third Edition (2006) pp 757-795
- [20] Warren J. Smith
Modern Optical Engineering
McGraw-Hill
Fourth Edition (2008) pp 175-204, 380-381

