[width=]"Figures/opticaldesign"

Figure 1: Optical design of the new SPIM microscope. Straight black lines represent mirrors; arrowed lines represent lenses; coloured lines represent laser light.

1.5
455nmRGB0,97,255 488nmRGB0,247,255 561nmRGB198,255,0 647nmRGB255,0,0

0.1 Light Sheet Microscope Implementation

To realise particle tracking within light sheet microscope improvements upon the previous system were considered and the entire microscope was to be rebuilt using a new design. Any optical development project comprises of a series of sub-systems, modules which can be built and designed so that the overall scale of the project is made more manageable. A microscope of this nature will have typically four aspects:

- Sample Holding
- Illumination
- Detection
- Image Acquisition

Though each section may somewhat affect the others key decisions and debugging can usually be localised to each. This section will discuss the new design, improvements upon the previous design and the current build state of the microscope for each of these components of the new system.

0.1.1 Sample Mounting

SPIM is notorious for sample mounting within the microscopy community, this is due to having a secondary excitation objective within close proximity to the detection objective. The concept of the inverted SPIM [**?**] allows more freedom in terms of sample mounting as more of the image volume is accessible. This design point is one of the axioms of the new system, the focus of this report.

The system was designed usually 45° **C**omputer **A**ided **D**esign so that two objectives would be mounted on 45° rails, allowing a detection and excitation objective to be lowered into a imaging space below. Each rail comprised of two 16 mm to 8 mm caging conversion mounts. The 16 mm mounts were attached to the rail by two guides placed in parallel to ensure that the objectives were in the same plane and correct angle. The objectives were held on 8 mm caging which attached to a thumb-screw mount holding each objective, so that they could both be very precisely lowered for fine adjustments. The 8 mm caging also carried an aluminium cube suitable for dynamically lowering a beam splitter or mirror into the system so that issues on excitation could be diagnosed, as the diverted light would be the exact the image of the back aperture of the excitation objective. Each rail was then mounted on an optical breadboard, with a noise dampening core to limit vibrations.

To allow the inverted objectives access to the samples the optical breadboard was cut with sufficient tolerance to allow them through but maintaining their stability; too large of a gap would cause sag in each arm, too small would impede access to the sample below. The hole was calculated using precision Solidworks, see Figure 0.1.1. This breadboard was then elevated to a height of 50 cm, using $25mm$ diameter aluminium posts for stability and vibrational dampening. The posts were mounted on a secondary optical breadboard which supported excitation optical components. The secondary breadboard was implemented with the ambition that the entire microscope could be "portable" as it did not have a permanent residence where it was being built. Hardware as well as a digitally controlled XYZ stage was placed on the bottom breadboard.

XYZ Stage

A Prior Pro Scan *HLD117* XY stage was mounted atop a Motorized Linear Axis *FB204E*. The set was chosen because of its suitable resolution due to the set having integrated linear encoders (20 nm in xyz); speed $300\,\mathrm{mm\,s^{-1}}$ in xy and $15\,\mathrm{mm\,s^{-1}}$) in z; large travel range ($120\,\mathrm{mm} \times 72\,\mathrm{mm}$ in xy and 38 mm in z) and versatility in terms of mounting onto the microscope and sample mountings and their ability for computer interfacing [**?**].

Comparison with old SPIM

The original SPIM design also used a dual inverted layout, but crucially, its detection and excitation arms were not as well seated. Each arm was held orthogonally to gravity, meaning each acted like a cantilever, bending unstably and unpredictably under its own weight. This moment meant that the system was less stable than the new design and very susceptible to vibrations, which add systematic noise that can not be corrected for. The old SPIM also lacked in that it did not have any motorised XYZ translation, this meant that is could not achieve large area imaging, high-throughput experiments nor could it facilitate single particle tracking in thick samples.

0.1.2 Illumination

Four typical laser sources were chosen to allow good specificity, future-proofing and overall versatility in the microscope. Wavelengths 455nm455 nm , 488nm488 nm , 561nm561 nm and 647nm647 nm were chosen as they are very typical fluorescent exciters of commercially available fluorophores in the visible range. The output power of the lasers (100 mW) is sufficient for good contrast images in SPIM, as it is within the single sun power regime. A beam quality M^2 nearing 1 is good, with $M^2 = 1$ being a theoretical ideal. Over the range of a metre the lasers will diverge by 1.2 mm, though this would double the beam diameter the sizes are appreciably small that it will have very little effect when corrected for through later optics. See Table 1 for a detailed specification.

They were combined into an overlapping beam using a set of standard dielectric mirrors and dichroic mirrors. Using two mirrors per beam allowed the degrees of freedom to accurately combine the beams in free space. The dichroic mirrors were chosen to ensure the each laser line propagated freely when approaching from behind the mirror but the correct colour laser was reflected appropriately; they were placed in the order e02 (dielectric as the first laser

[width=1]"Figures/solidworks$_design_front$"

Figure 2: Solidworks three dimensional representation of the 45^o inverted geometry allows sample access from beneath whilst still creating a fully orthogonal detection illumination system. Illumination comes from below on the right of the diagram, detection is normal to the page on the left.

Wavelength	455nm455	488nm488	561nm561	647nm647	±2 nm
Output Power	100	150	150	120	mW
M^2 (Beam Quality)	<1.2	<1.2	<1.1	<1.2	
Beam Diameter	0.6	0.8	0.7	0.9	±0.1 mm
Beam Divergence	<1.2	<1.2	<1.2	<1.3	mrad
Long-term Power Stability	<2	<2	<2	<2	% (8 h ±3 °C)
Laser Drive Modes	Continuous Wave, Analogue Modulation, Digital Modulation and Computer Control.				

Table 1: Significant information regarding the excitation laser emission sources.

does not combine with any others) zt594rdc, zt514rdc and zt458rdc. See Figure 3.

The alternative to using four lasers was having a single white light source and using the dichroic mirrors as long pass filters whilst using other filters to create the short pass. If this were the case then the power of each individual laser line would be dependent on the other laser lines. To then circumvent this an intensity modulator such as an **A**cousto-**O**ptic **T**unable **F**ilter would be needed as well. The former solution is more economical and more predictable. White light sources are expensive and do not produce homogeneous emissions, each colour may have been wholly variant in terms of quality for instance.

Structured Illumination

An SLM can be used in two modalities, either it can be imaged onto the back aperture of the objective or conjugated. When imaged onto the back aperture it directly manipulates Fourier space. The primary use of the SLM in this system was to create Bessel beam illumination, with that in mind it was decided that the SLM should be imaged onto the sample [**?**]. As Bessel beam needs only an annual ring in the Fourier domain, the majority of the light incident on the SLM would therefore be lost. For this reason the SLM will be imaging onto the sample directly using a minimum focal length of 47 cm, see Appendix **??** for details.

0.1.3 Light Sheet Generation

To generate a virtual light sheet a telecentric (sometimes called an f theta lens) is used to translate scanning mirror angle into a laser position shift creating a light sheet from a static laser beam. This lens couples with the tube lens to partially fill the back aperture of the objective so that a suitably thin light sheet is produced, with an ideal field of view tuned to compensate for the Gaussian profile of laser light. See Figure **??** for a schematic of light sheet generation.

Cage System

The light sheet generation components were all housed in cage system optomechanics as opposed to the free space optics of the bottom breadboard. The cage system was used as it projects the sensitive galvanometer mirrors as well as make lens alignment easier as the cage system *should* remove off axis by virtue.

Scan Lens

The focal length of the scan lens was initially unknown but its position for collimation was, and so a reasonable assumption of its focal length was possible. To compound certainty the focal length was found experimentally. To accurately measure the focal length of an unknown lens the focal length of a lens of known focal length is needed to collimate the light. In this experiment the tube lens of focal length 200 mm was used. By measuring the width of a laser beam prior (w_{before}) to and after (w_{after}) the collimating lens pair, the magnification is calculated very accurately and the unknown focal length is found by:

$$M = \frac{f_2}{f_1} = \frac{w_{before}}{w_{after}} \rightarrow \frac{f_2}{M} = f_1 \tag{1}$$

To measure a beam width very accurately a straight sharp edge is placed in the beam path and slowly iterated through, the resultant beam power is then measured using a power meter. To ensure there is no beam cropping on the power meter another lens was used to focus the intensity correctly, the same lens and its position was used in each measurement to keep with consistency. The beam power was measured and plotted which produces an integrated Gaussian profile (see (2)) otherwise known as an error function. Mathematically this is described by equation (3).

$$I(x,y) = I_0 e^{\frac{-2x^2}{w_x^2}} e^{\frac{-2y^2}{w_y^2}} \tag{2}$$

$$\begin{aligned} P_{TOT} &= I_0 \int_{\infty}^{\infty} e^{\frac{-2x^2}{w_x^2}} dx \int_{\infty}^{\infty} e^{\frac{-2y^2}{w_y^2}} dy \\ P(X) &= P_{TOT} - \int_{\infty}^{X} e^{\frac{-2x^2}{w_x^2}} dx I_0 \int_{\infty}^{\infty} e^{\frac{-2x^2}{w_x^2}} \\ &= \frac{P_{TOT}}{2} - \sqrt{\frac{\pi}{2}} I_0 \omega_y \int_{\infty}^{X} e^{\frac{-2x^2}{w_x^2}} \\ &= \frac{P_{TOT}}{2} \left[1 - erf\left(\frac{\sqrt{2}X}{\omega_x} \right) \right] \end{aligned} \tag{3}$$

Fitting of this curve was implemented using MatLAB's curve fitting package which utilises the method of least squares fitting, see Figure **??**. The fit result produced values of laser beam width as $w_{before} = 3.76 \pm 0.04$ mm and $w_{after} = 0.71 \pm 0.10$ mm. The value of w_{after} was supplied in Table 1 however, for posterity is was remeasured locally in case the value had changed or was incorrect. This gives a magnification M of 5.37 ± 0.10 therefore the focal length of the scan lens is 37.3 ± 0.1 mm. This also showed that the fill of the 12 mm

back aperture was 3.76 ± 0.04 mm hence the NA of the 0.3 objective used would be ≈ 0.094.

Back Aperture Fill

In the the old SPIM a standard NA of laser used was 0.15 which created a FOV of 520 µm [1] and a beam waist of 1.3 ± 1.0 µm (from equation **??**). To recreate a numerical aperture of 0.15 ± 0.05 , for an objective of focal length 20 mm the back aperture would need to be filled by a beam of diameter 6 mm. Therefore the excitation laser needs to be magnified 1.7×. Standard optical lenses are heavily quantised in terms of their focal length, a viable magnification for instance would be a 160 mm , 30 mm pair placed just before the flip mirror. A very sensible use of lenses would involve exploiting the lens that conjugates the SLM to the image plane, provided the lens were placed after the flip mirror and not before, which is possible if the focal length of the SLM is set higher than 330 mm and therefore placed after the flip mirror.

The beam waist was only measured for one colour though each colour may require its own additional lenses as well as the overall demagnifying telescope. These additional lenses directly after the laser heads would compensate for any width and colour dependent variances in the field of view of the light sheet; the gratuitous additional optics (six lens for three uncorrected colours) may make this solution inviable as the only correction it will achieve is likely a small improvement in light sheet homogeneity.

0.1.4 Detection

The optics surrounding the detection arm are far simpler than the excitation arm. The objective used is a planachromat 25× high (1.1) numerical aperture objective, to give good lateral resolution. This is coupled to another $ITL200$ tube lens which images infinity corrected emission light onto the Orca Flash 4.0v2 detector. Before the detector there is a Prior Filter wheel housing two emission filters from Semrock the $442/647nm$ Yokogawa emission filter and Chroma the $ET605/70m$. The emission filter is used to reject excitation light scattered from the sample, though this signal should be weak it is very difficult to correct for this digitally and so the filters are necessary.

0.1.5 Image Acquisition: Software Design

A well planned, modular and user-friendly interface is very important whenever creating hardware-user interfaces. In the case of the new microscope this software needs three different components. The first is the Camera control software which controls the detection arm of the microscope; this software needs to allow a user to save two and three dimensional stacks of images and still be accessible to change and upgrade as more and difficult biological questions are to be addressed. A controller for the stage that can interface with the camera software (to take images for large area scanning for instance) as well as being user friendly enough to accurately control the XYZ stage and finally, a waveform generator to control and synchronise the scanning mirrors, focus piezo and

[1] The chip size of the sCMOS camera is 13 mm and the magnification of the detection objective lens is 25× giving a field of view of 520 µm

potentially tunable lenses. These software also needed to be designed as modules so that a user could use each independent of the others. To satisfy these criteria LabVIEW was chosen as the programming environment to be used.

LabVIEW Architecture

LabVIEW is a graphical programming software package designed to produce simple, modifiable, user-friendly software in science. LabVIEW provides a wide library of drivers and libraries for hardware interfacing in science. Due to LabVIEW's modularity and fast development environment it is ideal for producing software for a microscope under development as any new hardware can be quickly and easily implemented and not necessarily by the original software writer.

Queuing

When interfacing with hardware a key challenge programmers face is how to mitigate command flooding at the device. A user may for instance want to send several commands in a linear fashion, but the device may only be able to successfully implement one or none if it crashes with overload or misinterprets the command as it is processing another. LabVIEW offers a queueing architecture where a programmer can send a command into a queue which will operate something, be it a real device or a construct within the software. This queueing opens a myriad of possibilities. The programmer can not only queue an infinite line of commands but also remove duplicate commands, truncate a command queue, change the routine of operation to a first in first out (FIFO) routine rather than first in last out (FILO) or even enforce that only one command can exist it the queue at any time.

Simple State Machine

A state machine is a fundamental computing architecture which can contend with a great deal of complex programmatical challenges. A common example of a state machine is a vending machine: when left alone the vending machine keeps its state (usually an enumerated variable) as idle. If a user adds a coin it begins a routine of *add currency to running total*. If a user presses dispense the next state will check the running total of currency, if the currency is too low the current state then changes its next state to return coin or any other operation. It is a very simple method of keeping a clear routine within the program rather than any abstractions that would occur if programmed otherwise.

Producer Consumer

In LabVIEW the combination of queueing and state machines facilitates the producer consumer concept, whereby the producer is an independent parallel routine which can queue commands for a consumer. The most simple template for this is an event driven producer recommended as the start of any LabVIEW routine, whereby the event loop collects commands from the Graphical User Interface (the front panel in LabVIEW). This producer then produces commands to be sent to the consumer loop which processes them. This ensures that command flooding and race conditions are avoided and the consumer loop can be self regulating. Crucially, queued commands can come from more than one entity in

this style of environment. Not only can commands from the front panel control the consumer, but also commands (if correctly written) can arrive from sources such as the internet, other devices, or other front panels within LabVIEW.

Camera.vi

Camera.vi was designed to be the main running loop of the software environment. That said, all of its functionality has been designed so that each function could be addressed by properly accessing the queue named **Camera**.

Front Panel

Care was taken to ensure that the user experience of the Camera.vi was well received and fundamentally simple. A simple user experience can both necessitate the microscope to be used by a lay individual for simple exercises as well as allow experienced users to access more advanced aspects of the microscope. The imaging area of the Camera.vi was therefore set within a scalable box, so that setting on the left and image recording settings would shift away. The settings on the left were organised into categorised tabs and initialised with camera defaults so that a complicated set up procedure is unnecessary. See Figure **??** for depiction.

Back Panel

The camera itself is split into three states: **init**alisation, **active** and **deinit**ialisation. This structure was used so that collaborators could easily contribute as required. Within *active* the full producer consumer architecture runs. The top loop typically seen as the producer takes all the front panel commands possible, passing the commands with any relevant numbers or information) in a bundled structure) to the camera control consumer loop. This loop then updates the camera as required and passes any images it received to a third slave loop which displays images as well as saving them to disk. This secondary loop uses a limited queue so that is for any reason LabVIEW cannot handle the output of the camera then frames will drop when in preview mode to compensate. Two disk saving modes were implemented in case of extreme uses of the camera.

The first mode extracts camera data directly to a temporary file on the hard drive (a $300\,\mathrm{MB\,s^{-1}}$ solid state drive 1 TB raid, which is typically sufficiently quick for standard operations) in an proprietary format which is later decrypted by the slave loop in parallel to the camera loop producing the files. Each temporary file contains a simple stack of images, designed such that each stack would be a full three dimensional scan of a volume. To avoid overwriting, each temporary file was automatically named by a number with a six digit upper limit, far beyond the limitations of the hard drive capacity.

The second mode records camera data directly to the computer's RAM which has much faster write speeds but is more limited in capacity to 32 GB in the current machine.[2]

[2]For the camera to interface with the older software which controls the waveform generation it needs to have the same LabVIEW environment. Unfortunately the old code was written in 32 bit LabVIEW with a Mathscript module that has no 64 bitt option as of yet. This means that the waveform generation software will need to be completely rewritten if the RAM writing record mode is ever fully needed; 32 bit systems are limited to 2 GB RAM considering the camera can output $600\,\mathrm{MB\,s^{-1}}$ this would overfill very quickly. That said, the record to harddrive function can run perfectly well in a 32 bit environment. Horses for courses.

[width=0.7]Figures/Excitation

Figure 3: Transmission Profile for Dichroic mirrors used demonstrating that each mirror will reflect only the wavelength intended.

Virtual Slit

The *Orca 4v2* has this slit scanning mode embedded in its firmware and is accessible from the supplied software. Inducing this within LabVIEW requires sending values to specific hex addresses using *advanced_property* functions. Two modes were implemented, a manual mode for debugging purposes, and an automated mode which reads the current input to create a suitably synchronised rolling shutter, see Figure **??**.

XYZ_Controller.vi

The Prior controller interfaces with software via an RS232 protocol. Provided with the stage is a library of routines within LabVIEW that package the correct RS232 commands in a LabVIEW friendly format. These were then incorporated into *XYZ_Controller.vi* producer consumer loop. This time however the stage controlling consumer loop was a slave to the consumer that handled front panel inputs. This was because the controller is centered about the list of positions. This list sends positional commands to the stage, but also requires functions such as: clearing the positional list; adding current position to the beginning or the end of the list; controlling the delay between positions and sending save image volume commands.

Waveform Controller

This module of the LabVIEW controller is the most crucial as it is the driver and synchronisation of the signals controller the scanning mirrors, focus stepper and tunable lens system if it is implemented. This module will also serve as an advanced calibration routine for the microscope to control.

Signal Train

When creating the virtual light sheet the most intuitive signal to send would be a high frequency triangular waveform with a peak-to-peak voltage equal proportionate to the field of view of the image; however, as a mirror on a galvanometer scanner is a relativity large inertia mass, the peaks and troughs of the waveform would not only over shoot but also cause excessive stress and potentially break the scanner. To circumvent this issue the mirror is returned to its base position sinusoidally, after each field of view scan, as the gradient of a sinusoid at its own peak is zero moving smoothly to zero again at its trough.

It is unlikely that the virtual light sheet will be perfectly flat relative to detection, to this end the virtual light sheet can actually be twisted by synchronising the z scanning mirror to the virtual sheet mirror tilting it by a gradient. To create a full three dimensional scan the z scanning mirror is offset along with its gradient step wise as seen in Figure **??**.