

# **Chapter 3**

## **Light-sheet microscope design and considerations**

This chapter introduces the instrument which was built and used during this thesis. It was required that the system presented here facilitated a wide range of imaging challenges, with two specific imaging tasks to be performed. The first task was to image Zebrafish embryos ( $\sim 500 \mu\text{m}$ ), surrounded by a magnetic tweezer system, during the 1k cell stage of embryo development (see Chapter 2); this was for the study of developmental mechanobiology. The second required the imaging of live (SHSY5Y) mammalian cells ( $\sim 50 \mu\text{m}$ ) for viral particulate tracking; this was for the study of 3D-live-cell viral egress using SPT.

## Specification

To facilitate these biological aims the system has to address these specification key criteria:

1. Fast volumetric imaging, 1 volume per second for the pixel range:  
 $2048 \times 2048 \times 100$
2. Multi-colour interlaced volumetric imaging
3. Capacity for multiple methods of sample mounting
4. Multiple imaging length scales, FOV range :  $200 \mu\text{m}$  to  $700 \mu\text{m}$
5. Options for exotic illumination development through optional illumination paths to incorporate an Spatial Light Modulator (SLM)
6. User-friendly and extensible software scheme in LabVIEW

## 3.1 Hardware

The design presented here is an adaptation of a previous light-sheet microscope, whose entire optical assembly was mounted on a set of rails rotated at  $45^\circ$ . The upgrade and redesign of the system was made such that the illumination and detection paths of the new system could be extended so that liquid tuneable lenses could be mounted in the vertically to reduce gravity induced optical aberrations.

The new design presented in this thesis adapts a previous design. For precise tracking of single virions, a more stabilised method of suspending the microscope objectives was needed as this minimises vibrational blur. This was problematic in the previous design due to design using cantilevered detection optics away from the frame, causing general instability.

### 3.1.1 Mechanical Design

The mechanical design of the light-sheet microscope consists of two optical breadboards, mounted one above the other. One for mounting the light sources (illumination board) and the second (imaging board) for the illumination and detection arms. The imaging board was mounted vertically above the illumination board on

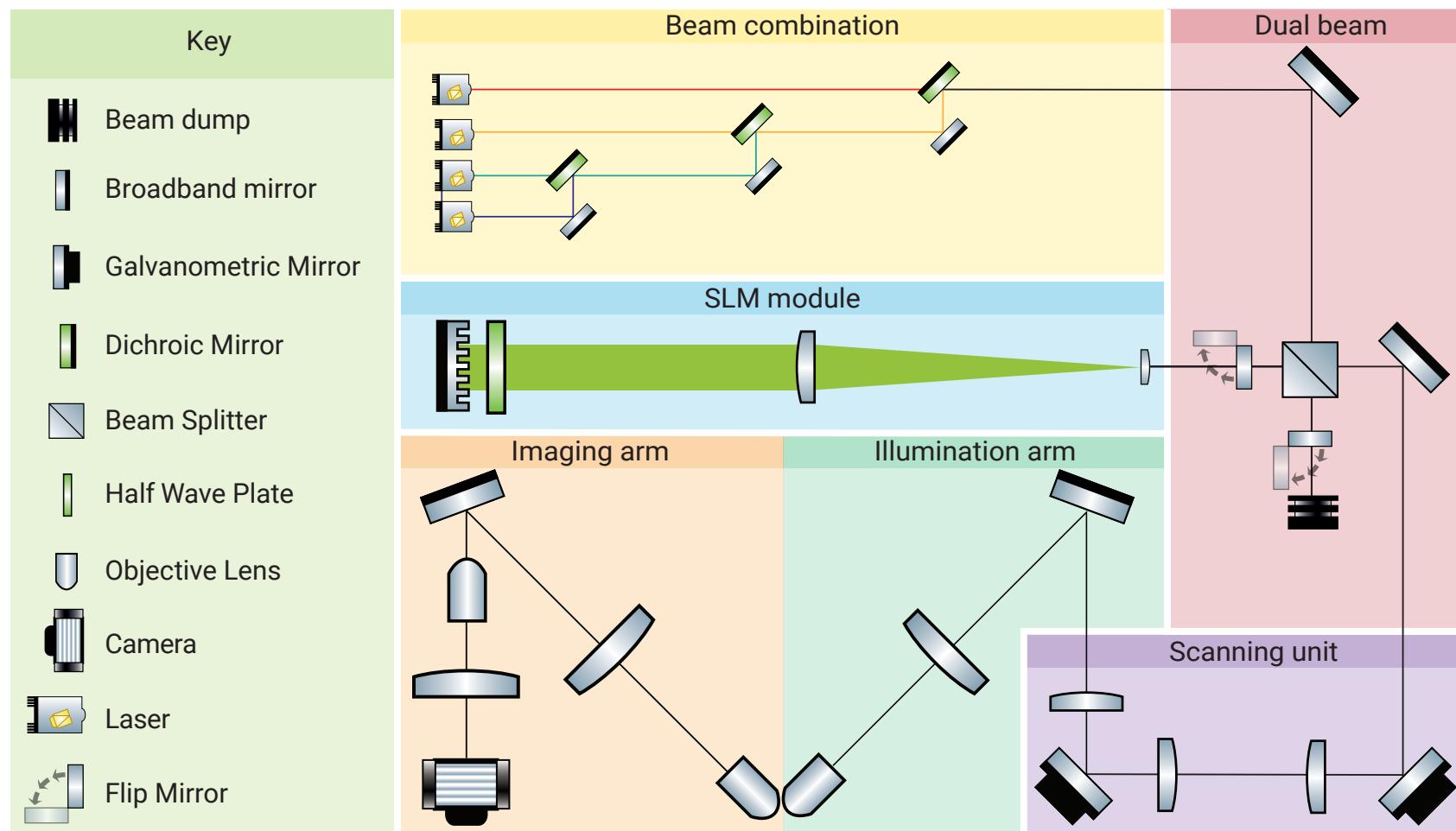
50.8 mm stabilising metal posts, which ensured the system was transportable as well as robust against vibrations. A large rectangular section of material was removed from the imaging board to allow the objective lenses to reach down to the samples inserted from below; too large of a gap would lead to excessive sag and vibrations in each arm, too small would impede access to the sample below the imaging board. The objective lenses and detection objective actuator were mounted on rails at 45° to the imaging board, the illumination and detection arms. These rails guided the objectives through the open section until the focal points of each objective (detection and illumination) met. The filter wheel was placed on the imaging optical rail before the camera and after a turning mirror on the detection arm so that the vibrations, due to filter switching and the camera fan, would be mostly decoupled from the detection optics. The camera was mounted at 45° on a Three Dimensional (3D) printed mount that corrected for the turning mirror on the imaging optical rail. Rotating the camera was necessary as it allowed the camera shutter and illumination beam to propagate concomitantly for slit-scanning, see later Chapter ??.

For the illumination path, a 22.5° mirror (to the horizon, see Fig. 3.2 (d)) was used to deliver the laser illumination from the scanning optics into the illumination objective on the illumination arm. The additional mirror was needed so that the scanning optics could be mounted flat to the optical breadboard, making positioning and optical mounting simpler. Using two mirrors also provided the sufficient degrees of freedom to align the axis of the scanning optics to the optical axis along the illumination arm. Finally, the light-sheet generating mirror of the scanning pair was suspended off the edge of the breadboard for delivery of the illumination from the bottom optical table, see Fig.3.2 (a).

## Sample Mounting

Sample mounting using light-sheet microscopes can be challenging due to the need to place a secondary objective (for illumination) within close proximity to the detection objective. The concept of the inverted Selective-Plane Imaging Microscope (iSPIM) [Wu2011] allows more freedom in terms of sample mounting as more of the image volume is accessible. The imaging board was mounted 500 mm above the illumination board so that an XYZ translator, with a large axial range, could be mounted. This allowed for multiple potential sample mounting strategies below.

**XYZ Stage** A *Prior* Pro Scan *HLD117* XY stage was mounted on top of a Motorized Linear Axis *FB204E* Z stage. The set was chosen as each component had integrated linear encoders, giving a suitable positional resolution (20 nm in  $xyz$ ); speed 300 mm s $^{-1}$  in  $xy$  and 15 mm s $^{-1}$  in  $z$ ; large travel range (120 mm  $\times$  72 mm in  $xy$  and 38 mm in  $z$ ). Each component was also computer controllable, and an open-source LabVIEW routine was developed and made freely available for this purpose [1].



**Fig. 3.1** Optical design of the light-sheet microscope. **Yellow:** Beam combination of the four laser lines using dichroic mirrors. **Red:** a beam splitter is used to create two beam arms which can be used together to create two parallel beams; one beam for single beam light-sheet; or one beam structured using the SLM (blue). **Blue:** Expansion optics for the optional SLM arm of the illumination system. **Purple:** A pair of scanning galvanometric mirrors are relayed onto each other and passed through a scan lens. **Green:** The illumination arm where the scanning beam is demagnified onto the sample. **Orange:** The high NA detection optics magnify the sample onto a second magnifying relay system and finally onto the camera.

### 3.1.2 Optical Design

### 3.1.3 Objective lenses

A ( $10 \times 0.3NA$ ) Nikon water-dipping objective was used for illumination and mounted at right angles to a ( $25 \times 1.1NA$ ) Nikon LWD water immersion objective. The water dipping objective used here has a 3.5 mm WD and narrow physical profile; meaning that when matched with the bulkier high NA detection objective there would be mechanical overlap if collimated light was coupled into the illumination lenses. Instead, the illumination tube lens supplied slightly diverging light to the back aperture of the illumination objective, which was found to efficiently resolve the challenge of separating the detection and illumination objective lenses. Very few objective pairs maximise detection and illumination NA whilst being water dipping and compatible, and a solution the solution Chapter ?? was found to be effective for imaging. A piezo scanner (Physik Instrumente *P-726 PIFOC high-load objective scanner*) was used to manually move the detection objective to match the detection focal plane to the illumination plane.

### 3.1.4 Illumination

Four laser sources were chosen to allow good specificity across the visible spectrum as well as for multi-colour imaging. Wavelengths **455 nm**, **488 nm**, **561 nm** and **647 nm** were chosen to excite typical fluorescent excitors of commercially available fluorophores in the visible range. The output power of the lasers (100 mW) was sufficient for good contrast images in SPIM.<sup>1</sup> The beams were combined using dichroic mirrors (Chroma *zt594rdc*, *zt514rdc* and *zt458rdc*) and broadband dielectric mirrors, the illumination setup is illustrated in Fig. 3.1.

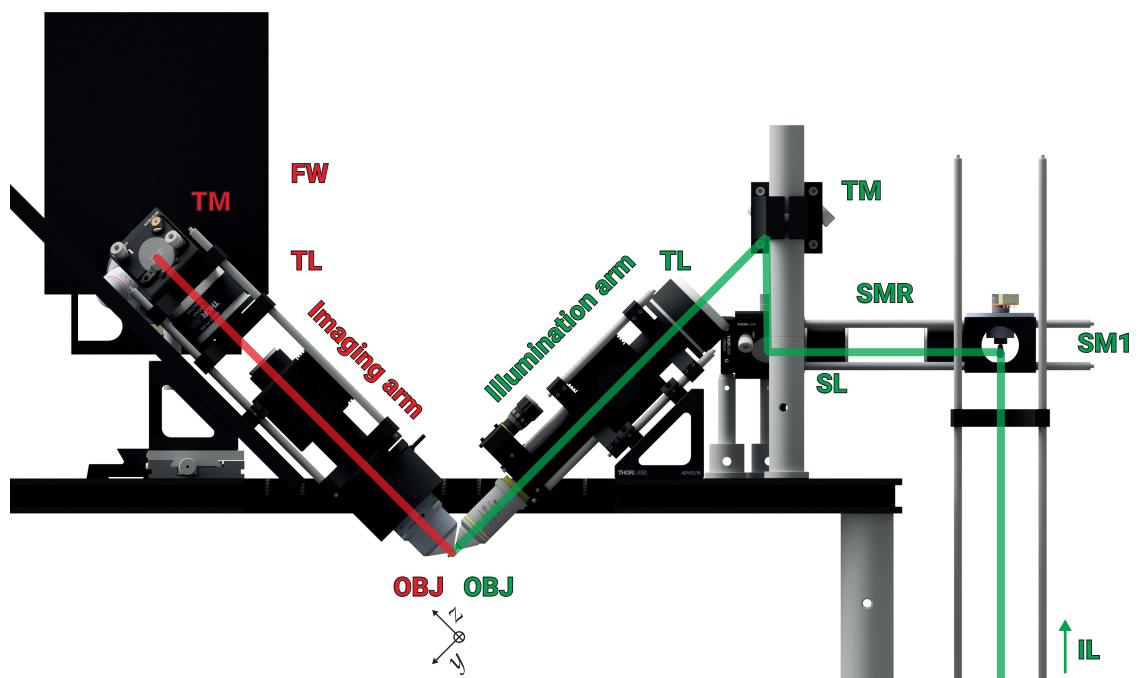
An alternative to using independent laser lines is using a white light laser source with chromatic notch filters. To modulate the power for each channel would require fast intensity modulation potentially using an Acousto-Optic Tunable Filter (AOTF). White light sources (e.g. Fianium SC390 supercontinuum lasers) are expensive; do not produce homogeneous emissions; are more costly as well as the class 4 laser beam being intrinsically more hazardous than the class 3b diode laser in this design.

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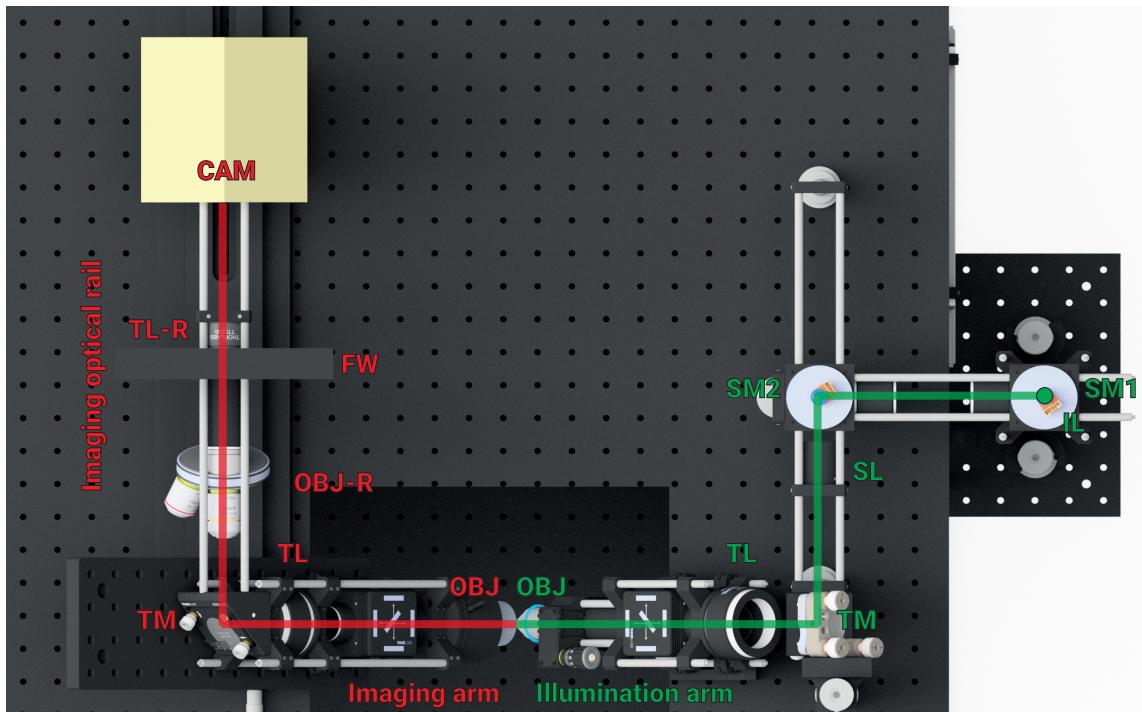
<sup>1</sup>SPIM is within the single sun power regime

### 3.1.5 Light-sheet generation

For generation of the light-sheet a galvanometric scanning mirror (*Cambridge Technology*) was placed behind a telecentric scan lens. The telecentric lens converts incident angle to emitted position such that a scanning mirror placed on-axis one focal distance behind the lens will produce a paraxial sweeping beam. The light-sheet generating scanning mirror was conjugated using a pair of matched lenses, in a 4f configuration, on a second scanning mirror. The second mirror was mounted at 90° to the light-sheet generating mirror to allow the sheet be displaced move axially with respect to the imaging lens. This allowed for fast volumetric imaging as well as correction for distortions caused by the scanning optics, discussed further in Chapter 3. Fig. 3.2 presents a schematic of light sheet generation.



(a) Computer Aided Design (CAD) model of light-sheet microscope from the side, gravity is down



(b) CAD model of light-sheet microscope from the top, gravity is going through the page

**Fig. 3.2** CAD three dimensional representation of the  $45^\circ$  inverted geometry imaging breadboard. Sample access is allowed from beneath whilst still creating a fully orthogonal detection and illumination system. The coordinate system was chosen with the imaging axis being coaxial with the the axially direction ( $z$ ) in the imaging direction, and right-handed with the  $x$  direction into the page and  $y$  in the direction of propagation of the illumination.  $\uparrow$ **IL**: Illumination comes from below on the right of the diagram. **SM1**: Scan mirror, generates the light-sheet. **SMR**: Scan mirror relay, optically relays image of **SM1** onto **SM2**. **SM2**: Scan mirror, moves the light-sheet axially. **SL**: Scan lens, keeps the light-sheet par-axial at the specimen plane. **TM**: Turning mirror ( $22.5^\circ$ ), redirects the light-sheet into illumination arm. **TL**: Tube lens (ITL200), focuses collimated illumination light into the back aperture of the illumination objective **OBJ**. Objective lens, for illumination.

**OBJ**: Objective lens, for imaging. **TL**: Tube lens (ITL200), images specimen plane to an image plane **TM**: Turning mirror ( $22.5^\circ$ ), redirects the emitted light into imaging optical rail. **OBJ-R**: Objective relay, on a turret for choosing multiple magnifications. **FW**: Filter wheel, for rejecting the non-fluorescent signal. **TL-R**: Tube lens relay, for imaging the relayed image plane onto the camera (CAM). **CAM**: Camera (sCMOS, Hamamatsu Orca Flash 4 v2.0), for detection of the fluorescent signal.

## Light-sheet shaping

Using the Orca Flash v4 camera (with a sensor size of 13 mm × 13 mm) and a detection objective magnification of 25 ×, produced a FOV with an extension of 520 µm in the  $x$  and  $y$  directions. To match the confocal width of the illumination beam to the FOV of camera sensor the illumination required 0.15 NA at the back aperture of the illumination objective for 561 nm light; providing a beam waist (light-sheet thickness) of  $1.3 \pm 0.1$  µm, from Equation ???. To create an NA of 0.15, for an objective of focal length 20 mm, the back aperture would need to be filled by a beam of diameter 6 mm.

Using an *ITL200* tube lens with a *Nikon A1* scan lens provided 5.37 × magnification (See Appendix ??), meaning the illumination objective back aperture would have been overfilled (7.52 mm beam diameter at the back aperture) and the usable FOV too small. To address this, an iris was placed at the back aperture of the illumination objective to allow for manual tuning of the NA. The downside of stopping down the back aperture, was that some light was discarded Light-sheet systems do not require large doses to function ( $\sim 1$  mW) and discarding a fraction of the light from the 100 mW sources was not found to impede imaging.

### 3.1.6 Detection

The detection lens used was a PlanAchromat 25 × high (1.1) NA objective, select the unparalleled lateral resolution for a water dipping lens that could fit the mechanical constraints of the light-sheet system. This was coupled to second *ITL200* tube lens which imaged infinity corrected emission light onto the Orca Flash 4.0v2 detector. In the path between the detection objective a Prior Filter wheel housing emission filters (Semrock the 442/647; Chroma the *ET605/70m* and *ZT405/488/561/647rpc*) was installed to reject scattered illumination light.

A further lens relay was added on the imaging optical rail after the detection arm turning mirror. The relay comprised a tube lens and two objective lenses on a rotating turret. This provided a par-focal solution for magnifying the imaging by 2.5 × (*Olympus MPLFLN1.25x*) and 1.25 × (*Olympus MPLFLN2.5x*) for 62.5 × and 31.25 × total magnifications respectively. Using microscope objectives for the additional optical relay ensured that there were minimal optical losses as the lenses are designed for the weak fluorescent signal, as well as for minimal distortion and chromatic aberrations. Imaging at 62.5 × magnification gives 99.2 nm lateral

sampling at the specimen plane. The Raleigh condition of the system, using 561 nm light, is 311 nm resolution. Therefore, at  $62.5 \times$  magnification, the system is sampled sufficiently for Nyquist criterion (155.55 nm) as the demagnified width of each photosite (from the camera, at the specimen plane) is  $\frac{6.5\text{ }\mu\text{m}}{62.5} = 104\text{ nm}$ .

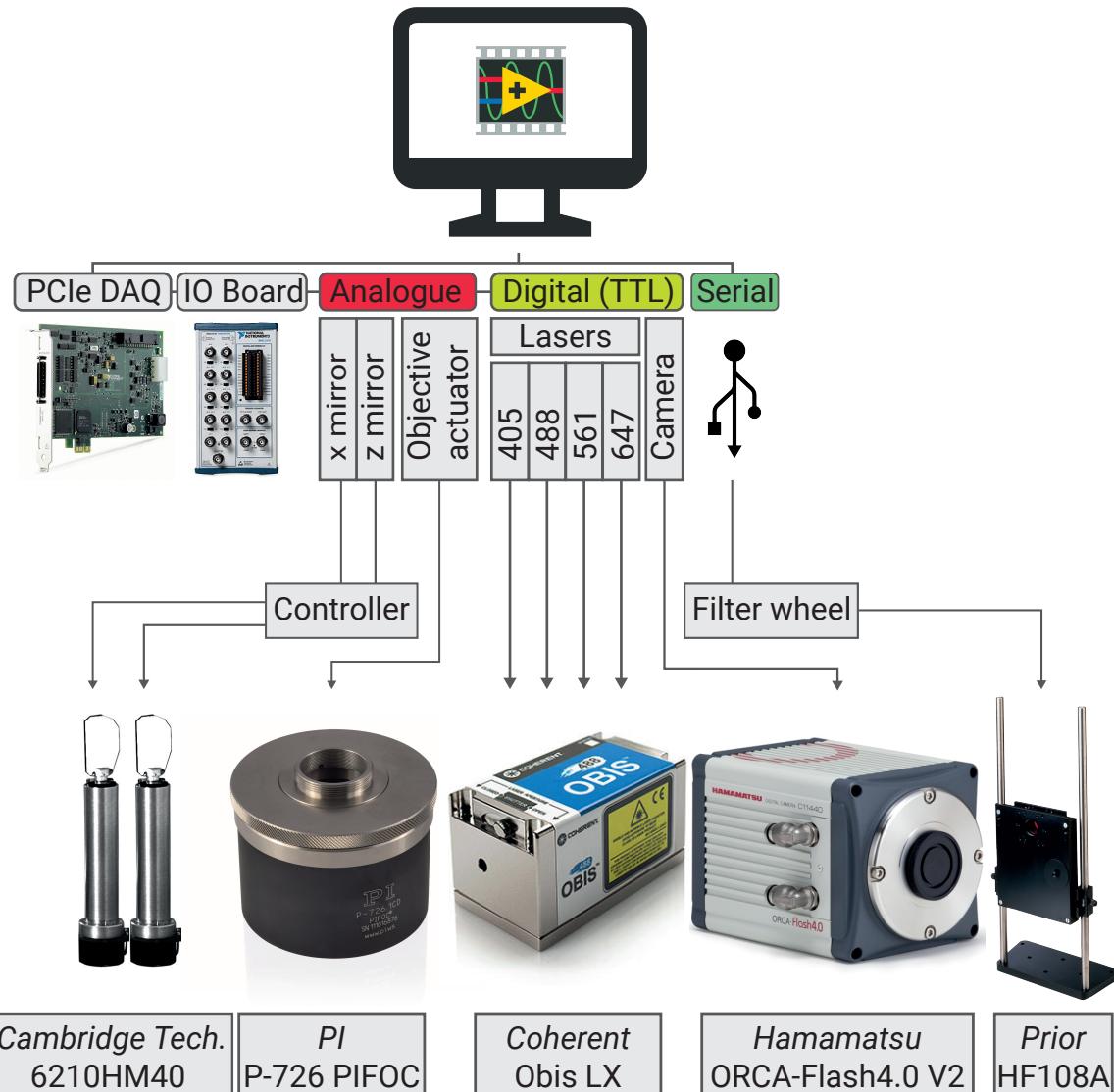
## 3.2 Software

To control the light-sheet microscope and acquire imaging data an open-source [1] software interface was developed, using LabVIEW, to send the appropriate electronic signals and serial commands.

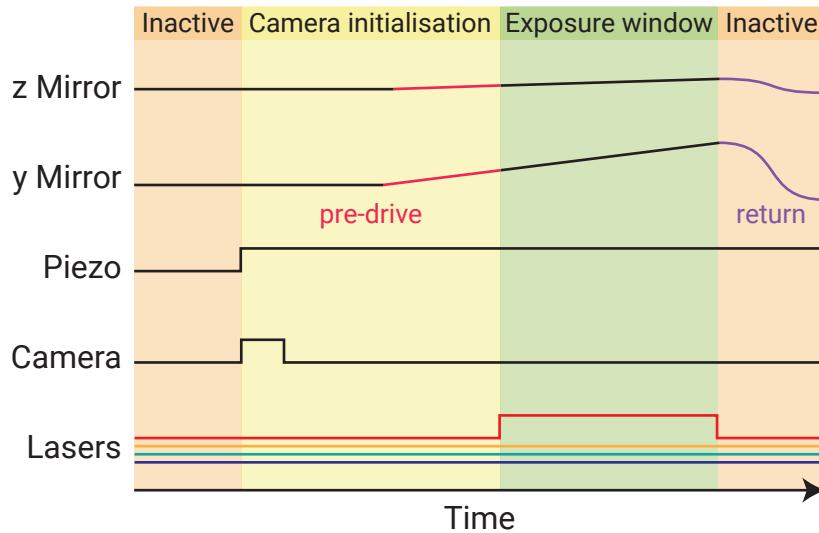
### 3.2.1 Signalling

Precise synchronisation is needed for confocal slit-scanning (Chapter ??) to be viable, as the time between the switching of active photosite rows at 100 Hz for the Orca Flash v4 is  $\sim 10\text{ }\mu\text{s}$ . This was achieved using fast electronics to send sets of packaged voltage waveforms through a National Instruments Data Acquisition (DAQ) module. Once a Transistor-Transistor Logic (TTL) 5V signal is sent to the camera, there is a delay of approximately 10 ms for the electronics to initialise on the camera. During this window the *y* mirror, which creates the light-sheet, and *z* mirror are pre-driven so that the mirrors are travelling at a constant velocity during the exposure window, giving a uniform illumination. The time-point of the start of the exposure window was found empirically (9.8 ms after camera triggering) by tuning this window until the illumination profile, under slit scanning, was uniform when visualised using fluorescent dye (Rhodamine). During the exposure, a TTL signal is sent to the requisite laser channel for illumination. Once the exposure window is finished, the *y* and *z* mirror is sinusoidally returned to the start voltage for the next exposure (see Fig. 3.4); sinusoidal ramping helps protect the mirror against inertia-induced damage.

Control and synchronisation of the filter wheel and XYZ translation stage was achieved using Recommended Standard 232 (RS232) serial commands, as precise timing was not needed and direct feedback on the status of the stage and filter was desirable.



**Fig. 3.3** Control schematic of the digitally scanned light-sheet microscope. All control signals are generated within LabVIEW and distributed to the components. Components requiring fast signalling (lasers, scanning mirror, objective actuators) are synchronised by sending pre-built packaged signal trains.



**Fig. 3.4** The signals required to synchronise a rolling shutter in a digitally scanned light sheet. A pre-drive phase for the *y* mirror is needed to ensure the illumination profile of the light-sheet is uniform. The camera requires time to initialise the electronics and so a delay period is added within which the pre-drive of both mirrors is performed. Both mirrors are sinusoidally returned to their start position ready for the next acquisition.

### 3.2.2 LabVIEW control software

A modular, extensible and easy-to-use software solution was needed to control the light-sheet microscope. LabVIEW, a graphical programming language with an emphasis on electronic systems control, was used. LabVIEW provides a wide library of drivers and libraries for interfacing; in particular the Orca Flash v4 has LabVIEW drivers for direct control of its reader functionality, including the requisite commands for confocal slit scanning.

#### LabVIEW Architecture

It was required that the components within the microscope were interfaced in a parallel manner as well as controlling and interfacing with each other (e.g. the XYZ controller triggering the capturing of a image volume). The software controller was engineered using a producer where each consumer was a parallel state machine. The producer will receive front panel inputs, then convert and pass those commands on to the consumers. Using a queueing architecture ensured that command flooding and race conditions are avoided, and the consumer loop can be self regulating.

The commands were packaged as a bundle. The first part being an enumerated type which changed the *state* of the consumer; the second being the necessary front

panel data which informed the consumer on any updates to the states (e.g. camera exposure). By using this queuing architecture, consumers can then communicate with each other whilst functioning independently.

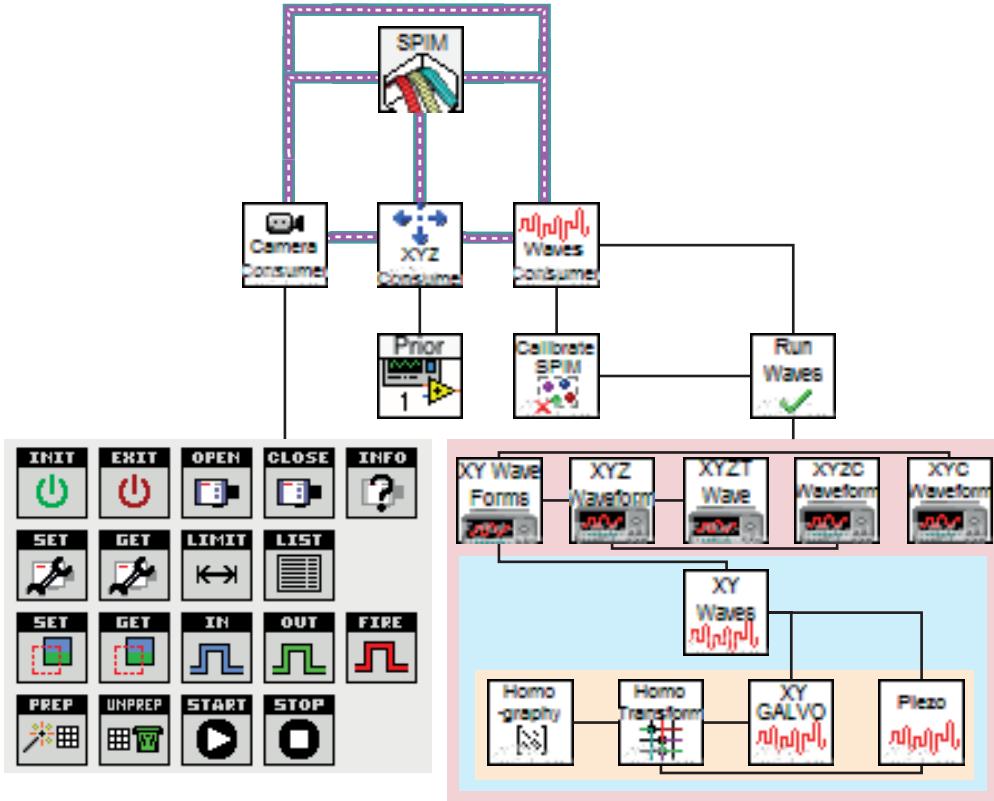
## Kernel

The kernel stores the acquisition settings and initiates all the queues to be sent to the sub modules, **Camera**, **Waveform Generator** and **XYZ Translator**. All acquisition settings and properties are converted into enumerated type enumerated state machine commands. Acquisition modes are organised into imaging orders, such as *XYZ*, *XYZC*, *XYCZ*, *XYT*; where *XY* is a single frame, *Z* is an iteration axially, *C* is the colour channels selected, and *T* is the time course selected. Each of the dimensions (*XYZT*) are governed by the parameters *Start*, *Step*, *Range*; where start is the initial position or time; step is the step size, meaning spatial resolution or temporal resolution; and range is the range over which the dimension covers, spatially or temporally. For colour, a  $2 \times n$  array of enumerated typed is constructed for laser line and respective filter wheel position choice. The kernel also stores the calibration coordinates (see Chapter 3) sent from the calibration module. All of the settings can then be exported as XML files and may be reloaded later.

## Camera module

The camera module consists of two consumers. The first consumer initiates and destroys the camera communications; as well as soft update states (for instance changes to exposure time), and hard update states (for when an acquisition setting requires the camera link to be unloaded, such as changing the *sensor mode* which controls the shutter directions). The difference between hard and soft updates is poorly documented and the categorisation was found empirically.

The second consumer loop exists within the camera module to handle saving and displaying of image data. This consumer has two settings: the first receives queued image arrays directly from the camera, this is used for live preview mode and small image sequences such as single volumes; the second reads and converts image files from the file-stream of the camera. The latter mode does not drop frames as the camera is streaming data directly to the hard drive, provided the read and write streams of the hard-drive do not overflow. The single frame acquisition mode



**Fig. 3.5** Schematic diagram of the dependancies of each routine in the LabVIEW software (SPIM, the kernel) that runs the light-sheet microscope. The *Camera consumer* and *XYZ consumer* package the Hamamatsu capture (grey) and Prior iScan libraries (Scan) respectively. The *Waves consumer* packages the signal generating routines (red) and drives the resultant signals to the DAQ board using *Run waves*. Each of the waves routines (red) are concatenations of *XY waveforms*, which itself relies on *XY waves* (blue) generates signal trains from calibration coordinates and front panel data. *Homo-graphy* and *Homo transform* (orange) take calibration coordinates to then inform *XY Galvos*.

The purple connections represent LabVIEW queue connectors; each purple-connected module, other than the main kernel (SPIM), was an independent queued consumer state machine. State changing commands were added to each module's queue and could be received from any other connected module. This allowed for conditions such as the *XYZ controller* being able to request *Camera consumer* for an image or volume to be recorded using a simple enumerated command.

will also not drop frames due to their sequence being queued, but, there may be a delay between the presented image and the live view at high frame rates.

**Virtual slit** The virtual slit for confocal slit scanning was addressed in the camera's hardware directly using hex address  $\times 400210$ . Mode 1, sets the camera to full frame and mode 12, slit scanning. Once the mode is set the line interval ( $\times 403850$ ) and slit exposure ( $\times 1F0110$ ) is set according to the equation:

$$\text{Slit exposure} = \frac{\text{Exposure} \times \text{Slit width}}{10 \text{ ms} + \text{FOV}_y + \text{Slit width}} \quad (3.1)$$

$$\text{Line interval} = \frac{\text{Slit exposure}}{\text{Slit width}} \quad (3.2)$$

## Waveform module

The waveforms module handles all signalling for the DAQ board (four laser lines, piezo, Y mirror, Z mirror, camera trigger, filter wheel). The waveforms are constructed from the acquisition settings and are forced to synchronise using propagation error values<sup>2</sup>.

The camera and lasers were addressed using digital (TTL) signals which are hardware limited between 0 V to 5 V. Voltages to the objective actuator and scanning mirrors were software limited between 0 V to 10 V and  $-10 \text{ V}$  to 10 V ( $10 \text{ V} = 100 \mu\text{m}$ ) respectively, to prevent damage to the electronics. The objective actuator was positioned linearly, by voltage, using the conversation  $10 \text{ V}^{-1} \mu\text{m}$ ; with 16 bit voltage resolution from the DAQ, this gave an addressable axial resolution of 1.52 nm, which was  $4 \times$  larger than the reported closed loop resolution (0.4 nm) of actuator. Achieving the full resolution would have required addressing the actuator using serial commands which would have been too slow for the required imaging modes, such particle tracking. As such the resolution trade-off was accepted.

## Calibration module

The calibration module set the microscope to live image preview mode with direct user control over the objective actuator voltage and mirror voltages. The module

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<sup>2</sup>LabVIEW is a data flow language so synchronisation is controlled using propagating variables and commonly using the error output of the function.

was used to set the limits of the usable volumetric FOV and match the focus of the objective to mirror positions, discussed in detail in Chapter 3.

### 3.3 Specification review

**1. Fast volumetric imaging** Fast volumetric imaging was achieved using a pair of optically relayed scanning mirrors to rapidly sweep a virtual light-sheet through volumes. A Piezo objective actuator was used to maximise the axial speed at which volumes could be acquired.

**2. Multi-colour volumetric imaging** Four laser lines were used with a 6-port fast filter wheel on the imaging optical rail and simultaneous TTL-control of diode laser illumination to allow for fast colour switching, to image multi-colour volumes rapidly. The limiting step for speed was the filter wheel, though multi-notch filters were used for bespoke cases needing maximal colour switching speed (in which case only the laser diodes were switched to change colour channels).

**3. Capacity for multiple methods of sample mounting** An XYZ translator was mounted well below the two dipping objectives. This allowed for traditional mounting strategies, such as agarose filled Fluorinated Ethylene Propylene (FEP) tubing, as well as bespoke solutions for difficult samples, such as live cells. The translator enabled precision positioning as well as large FOV imaging through positional mosaicing. Chapter ?? discusses, in detail, the sample mounting procedures used.

**4. Multiple magnifications** A par-focal relay using microscope objective lenses was inserted in the detection path to allow for two FOVs to be chosen from. This allows for the imaging of a large gamut of biological samples, from the cell up to the organism.

**5. Options for exotic illumination development** A beam splitter was placed on the lower optical breadboard before reaching the scanning mirrors above. Using flip mirrors and beam dumps enabled the option of a dual-beam illumination or an exotic illumination from the SLM found on one of the arms.

**6. User-friendly and extensible software scheme** LabVIEW was used to create a modular system for the control software. By using an appropriate architecture, as detailed above, modules could interact freely and run in parallel.

The following chapter will expand on the signal generation presented in this chapter in an attempt to optimise and maximise co-planarity between the imaging and illumination planes.



# References

1. Russell, C. *Spim-Controller* <https://github.com/laseranalyticsgroup/spim-controller> (2018).