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 biological ~~aims to be met~~. The first ~~of which required the system to~~ image Zebrafish embryos (~500 µm), surrounded by a magnetic tweezer system, during development (see Chapter 9); this was for the study of developmental mechanobiology. The second required the imaging of live cells (~50 µm) for viral particulate tracking; this was for the study of 3D-live-cell viral egress using SPT.

3.1 Specification

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

(a) Fast volumetric imaging

(b) Multi-colour volumetric imaging

(c) Capacity for multiple methods of sample mounting

(d) • Multiple imaging length scale

(e) • Options for exotic illumination development

(f) • User-friendly and extensible software scheme

3.2 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°. The upgrade and redesign of the system was made such that the excitation and detection paths of the system could be extended so that liquid tuneable lenses could be mounted in the direction of gravity to reduce induced optical aberrations.

The previous design cantilevered the detection optics away from the frame, causing general instability. For precise tracking of single virions, a more stable suspension of objectives was needed.

3.2.1 Mechanical Design

The mechanical design of the system consists of two optical breadboards mounted one above the other on 50.8 mm stabilising metal posts. A large rectangle of material was removed from the upper board to allow the objective lenses to reach down to the samples inserted from below; too large of a gap would cause sag in each arm, too small would impede access to the sample below. The objective lenses and detection objective actuator were mounted on rails at 45° to the top motherboard. These rails guided the objectives through the open hole until the focal points of each objective (detection and excitation) met. The filter wheel was placed on an optical rail before the camera and after a turning mirror so that vibrations of filter switching would be mostly decoupled from the detection optics. The camera was mounted at 45°, on a 3D printed mount to correct for the turning mirror, on the same optical rail. Rotating the camera was necessary as it allowed the camera shutter and illumination beam to propagate concomitantly, see Chapter 6.

For the excitation path, a 22.5° mirror was used to to deliver the laser illumination from the scanning optics into the objective. 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 excitation arm. Finally, the light-sheet generating mirror of the scanning pair of was suspended off the edge of the breadboard for delivery of the illumination from the bottom optical table.

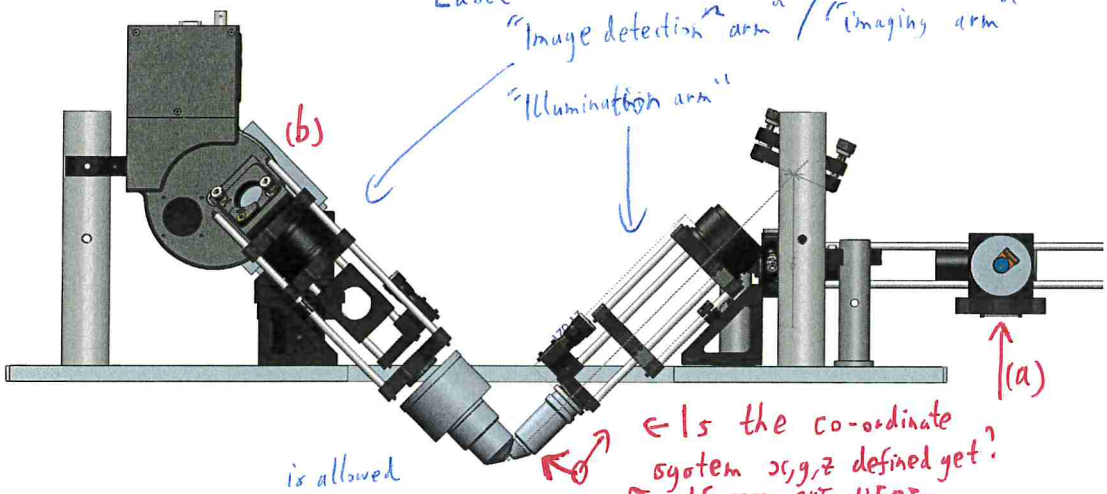


Fig. 3.1 Solidworks three dimensional representation of the 45° inverted geometry allows sample access from beneath whilst still creating a fully orthogonal detection illumination system. Illumination comes from below on the right of the diagram, detection is normal to the page on the left.

Sample Mounting

Sample mounting using light-sheet microscopes can be challenging 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. The upper optical table was mounted sufficiently high 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*. The set was chosen as each components 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 × 72 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 [?].

3.2.2 Optical Design

Fig. 3.2 (?)

3.2.3 Objectives

A (*10× 0.3 NA Nikon*) water-dipping objective was used for excitation and mounted at right angles to a (*25× 1.1 NA*) 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 bulky high NA detection objective there was a slight mechanically interference, which was corrected for using the tube lens to adjust the working distance. Very few objective pairs maximise detection and excitation NA whilst being water dipping and compatible, see Chapter 7. 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 excitation plane.

3.2.4 Illumination

Four laser sources were chosen to allow good specificity across the visible spectrum as well as for multi-colour imaging. Wavelengths 405 nm , 488 nm , 561 nm and 647 nm were chosen to excite typical fluorescent exciters of commercially available fluorophores in the visible range. The output power of the lasers (100 mW) was sufficient for good contrast images in SPIM. [1] The beams were combined using dichroic mirrors (*Chroma zt594rdc, zt514rdc and zt458rdc*) and broadband dielectric mirrors See Figure 3.2.

An alternative to using independent laser lines 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. White light sources are expensive and do not produce homogeneous emissions as well as the overall solution being more costly.

3.2.5 Light-sheet Generation

For generation of the light-sheet a galvanometric scanning mirror (*Cambridge Technology*) was placed behind a telecentric scan lens. The lens converts incident angle to emitted position such that a scanning mirrored placed on-axis a focal distance behind will produce a parallel sweeping beam. The light-sheet generating scanning mirror was conjugating 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

[1] SPIM is within the single sun power regime

Rephrase.. there would be a mechanical overlap of the lenses if the illumination lens were supplied with collimated light was used to coupled into the illumination lens. Instead, the illumination tube lens was specified to supply slightly diverging light to the back aperture of the illumination lens, which was found to efficiently resolve the challenge of separating the objective lenses.

non-italic

illumination (?)

ier

and this solution was found to be effective for imaging

Why are these italic.

The illumination setup is illustrated in Figure 3.2

Use whole sentences to refer clearly to figures. "See Figure 3.2" just feels rough "to a reader.

Ok stet

(e.g. Fianium SC390 supercontinuum lasers)

space

And the beam being intrinsically more hazardous than the class 3B diode lasers in this design.

class 4 laser

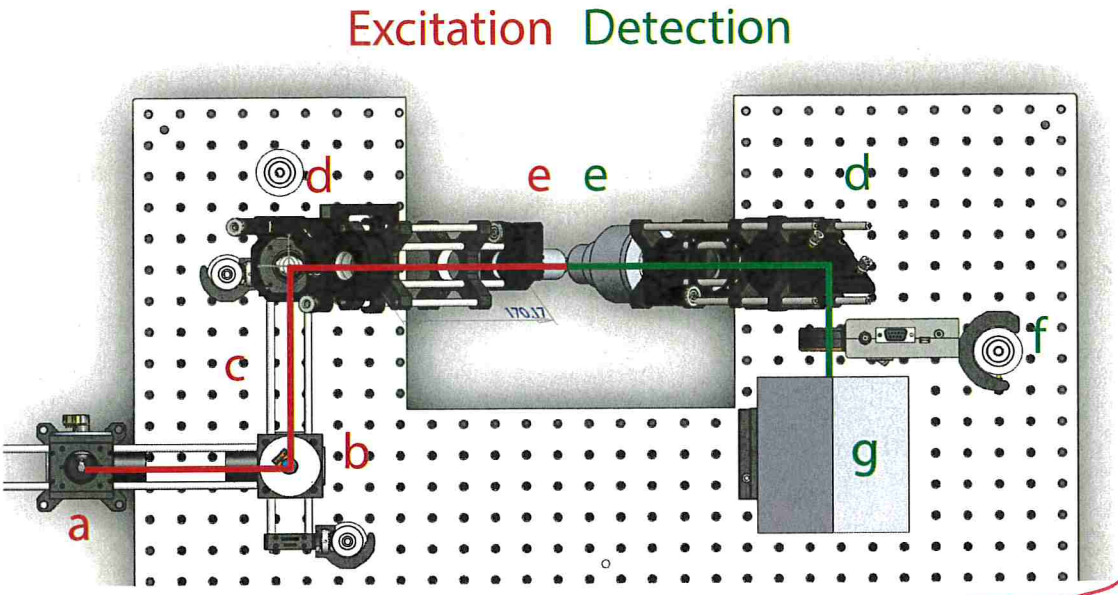
TELECENTRIC

one

BEYOND THE LENS

ED

paraxial (?)



Where is the co-ordinate system

Fig. 3.3 a) Scan mirror which creates the light sheet. b) Scan mirror in z for scanning volumes. c) Position of telecentric lens. d) Tube lens *ITL200* e) Objective lenses, with green having an objective actuator. f) Emission filter wheel g) sCMOS camera

be displaced with respect to the imaging lens.

generating mirror to allow the sheet to ~~move~~ axially in the volume. This allowed for fast volumetric imaging as well as correction for distortions caused by the scanning optics, see Chapter 4. See Figure 3.3 for a schematic of light sheet generation.

discussed further in

presents

DIAGONAL? GIVE, e.g. 9×9 mm if possible.

or 13×13 mm.

Light-sheet shaping

sensor.

Using the Orca Flash v4 camera (with a chip size of 13 mm) and a detection objective magnification of 25 ×, gave a required FOV of 520 µm. To match this to the confocal width of the propagating beam required 0.15 NA illumination for 561 nm excitation, providing a beam waist (light-sheet thickness) of 1.3 ± 1.0 µm, see Equation 1.6). To create a numerical aperture 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. Therefore the excitation beam needed to be magnified 1.7 fold.

Why "match". Reader won't understand.

~~based on~~ from Equation 1.6.

[of diameter 4mm after....]

Seems like jargon. Try ~~indicates~~ produces a field of view with an extent of 520µm in the x and y directions.

Using an *ITL200* tube lens with a *Nikon A1* scan lens provided 5.37 × magnification (See Appendix A.1), meaning the excitation objective back aperture would have been overfilled and the usable FOV too small. To address this, an iris was placed at the back aperture of the objective to allow for manual tuning of the NA. The downside of stopping down the back aperture, was that light was ~~being~~ discarded; ~~as~~ light-sheet

illumination.

some

systems do not require large doses to function (~1 mW), so discarding the majority of the light from the 100 mW sources did not impede imaging.

3.2.6 Detection

The detection lens used was a PlanAchromat 25× high (1.1) NA objective, giving unparalleled lateral resolution for a water dipping lens. This was coupled to second *ITL*200 tube lens which imaged infinity corrected emission light onto the Orca Flash 4.0v2 detector. Before the detector there was a Prior Filter wheel housing emission filters (Semrock the *442/647*; Chroma the *ET605/70m* and *ZT405/488/561/647rpc*) used to reject scattered excitation light.

A further lens relay was added on a optical rail after the 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 them having minimal distortion and chromatic aberrations. Using 62.5 × magnification gives 99.2 nm lateral sampling at the image plane. The Raleigh condition of the system using 561 nm light is 311 nm resolution. Meaning that at 62.5 × magnification, the system is sampled sufficiently for Nyquist criterion (155.55 nm) which states that the sampling of a system needs be twice the bandwidth of a band-limited signal.

3.3 Software

To control the hardware, a software interface was needed which would send the appropriate electronic signals and serial commands.

3.3.1 Signalling

Precise synchronisation is needed for confocal slit-scanning (Chapter 6) to be viable, as the time between the switching of active pixel rows at 100 Hz for the Orca Flash v4 is ~10 µs. This was achieved using fast electronics to send sets of packaged voltage waveforms through a National Instruments DAQ module. Once a TTL 5V signal is sent to the camera, there is a 10 ms delay for the electronics to initialise on

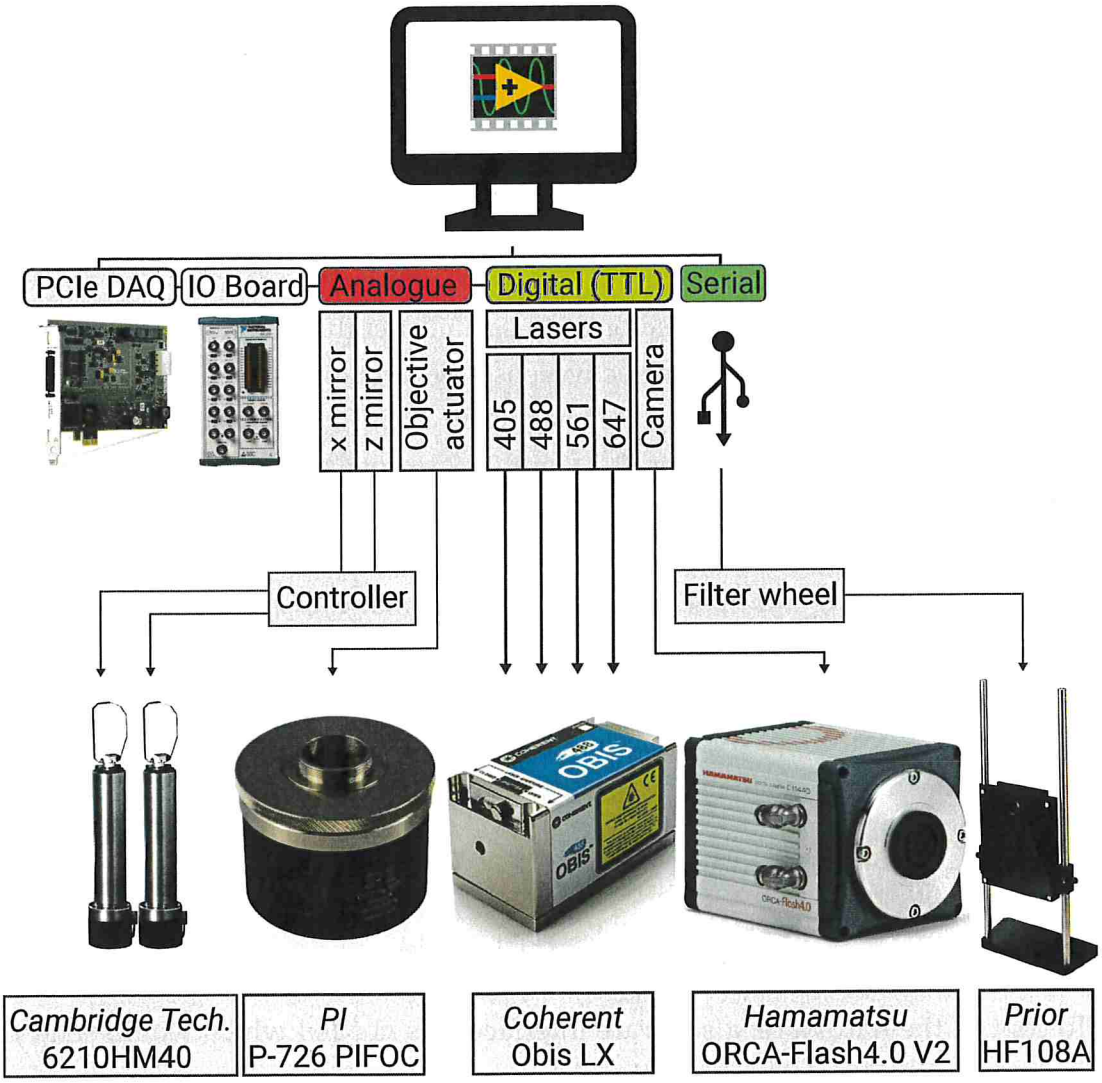


Fig. 3.4 Control schematic of the digitally scanned light-sheet microscope. All control signals are generated within LabVIEW and distributed to the components. Components requiring fast signalling (lasers, scanning mirror, objective actuators) are synchronised by sending pre-built packaged signal trains.

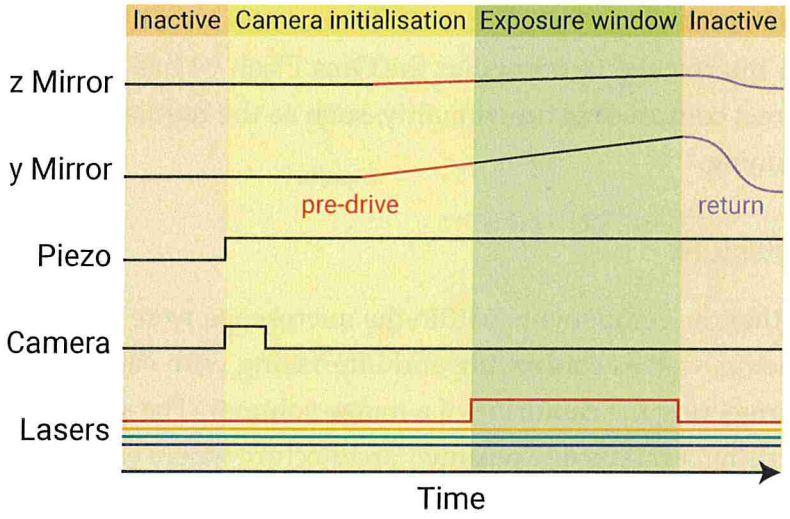


Fig. 3.5 The signals required to synchronise a rolling shutter in a digitally scanned light sheet. A pre-drive phase for the y mirror is needed to ensure the illumination profile of the light-sheet is uniform. The cameras requires time to initialise the electronics and so a delay period is added within which the pre-drive of both mirrors is performed. Both mirrors are sinusoidally returned to their start position ready for the next acquisition.

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 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 Figure 3.5); this helps protect the mirror against inertia-induced damage.

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

3.3.2 LabVIEW control software

A modular, extensible and easy-to-use software solution was needed to control the 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 deep and direct control over functionality, such as the requisite commands for confocal slit scanning.

LabVIEW Architecture

It was required that the components within the microscope were interfaced with 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 Consumer architecture 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 consumer *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 initiates all the queues to be sent the the sub modules, **Camera**, **Waveform Generator** and **XYZ Translator**. All acquisition settings and properties are converted into 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 4) sent from the Calibration module. All of the settings can then exported as XML files and may be reloaded later.

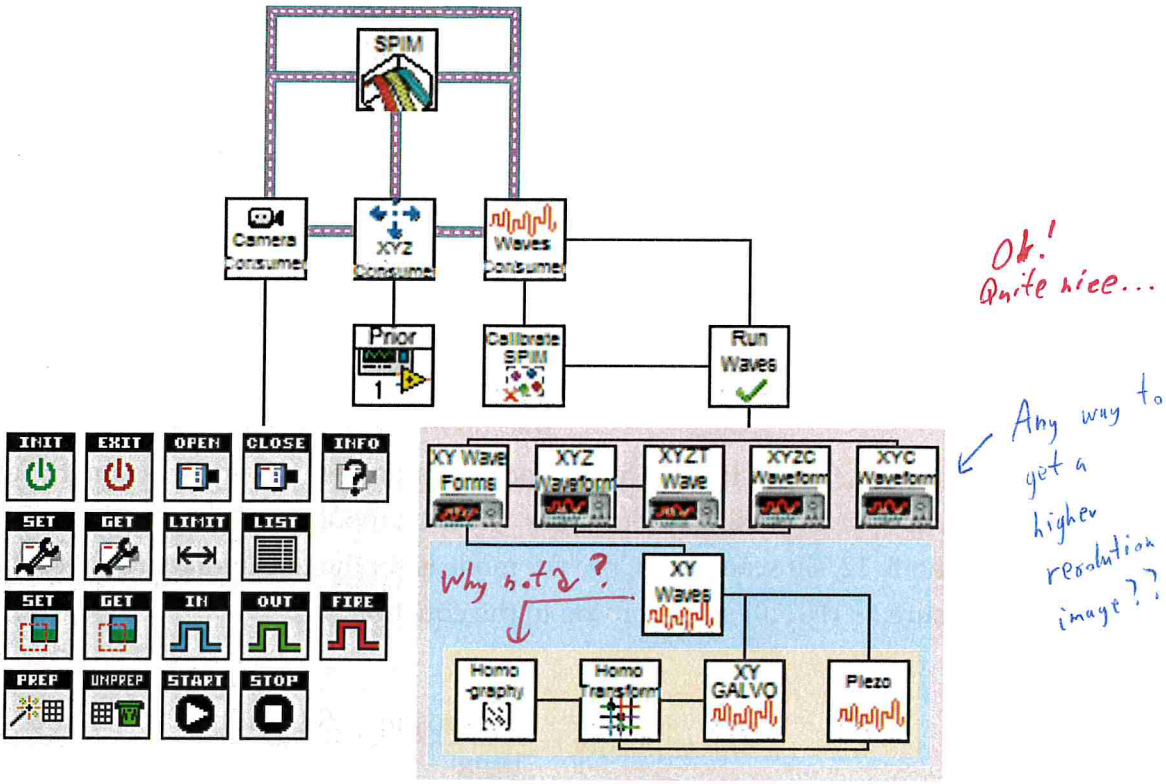


Fig. 3.6 Schematic diagram of the dependancies of each routine in the LabVIEW software (*SPIM*, the kernel) that runs the light-sheet microscope. The *Camera consumer* and *XYZ consumer* package the Hamamatsu capture (grey) and Prior iScan libraries (*Scan*) respectively. The *Waves consumer* packages 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*.

to generate

Camera module

The 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 to be unloaded (such as changing exposure). The additional consumer loop exists within the 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 are not overflowing. The single frame acquisition mode 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 ×400210, mode 1 sets the camera to full frame and mode 12, slit scanning. Once the mode is set the line interval (×403850) and slit exposure (×1F0110) set according to the equation:

$$\text{Slit exposure} = \frac{\text{Exposure} \times \text{Slit width}}{10\,\text{ms} + \text{FOV}_y + \text{Slit width}}$$

$$\text{Line interval} = \frac{\text{Slit exposure}}{\text{Slit width}}$$

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 for digital and analogue signals, which are forced to synchronise using propagation error values.

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 V to −10 V (10 V = 100 µm) respectively, to prevent damage to the electronics. The objective actuator was positioned linearly by voltage using the conversation $10\,\text{V}^{-1}$ µm; 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 too slow for 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 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 4.

3.4 Objectives review

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.

Multi-colour volumetric imaging Four laser lines were used with a 6-port fast filter wheel and the illumination lines were TTL-trigged diode lasers 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 available for bespoke cases needing maximal colour switching speed.

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 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.

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 allow for the imaging of a large gamut of biological samples, from the cell up to the organism.

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.

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.

Generally good.

Major comment – needs co-ordinate system defined somewhere.