An inverted fluorescence microscope assembly

1. Introduction

In certain instances it is desirable to construct one's own inverted microscope optical 'block'. One of the reasons is that the cost for commercial microscope bodies is high (and increasing, as more and more 'features' are added by the manufacturers). When dealing with fluorescence imaging (either widefield or laser beam scanning), the microscope body can be extremely simple since epi-illumination is most commonly employed and a transillumination condenser is not required. Moreover, eyepieces are not needed if camera-based image acquisition is used.

We describe here details of a system developed by our group. Several versions of this basic arrangement have been constructed and are in use in several laboratories. Although we have based the system around Nikon CF160 'infinity' optics, in principle optical systems from other manufacturers can be used. We chose Nikon because individual optical components (e.g. tube lenses) are readily available and because they are ready to lend components without requiring a 'sale'.

It is useful to remind ourselves of the basics of epi-fluorescence microscopes, and in particular the basics of Köhler illumination. When it comes to transillumination optical systems, Köhler illumination principles are well described in numerous textbooks; epi-fluorescence illumination is similar and is shown in Figure 1.

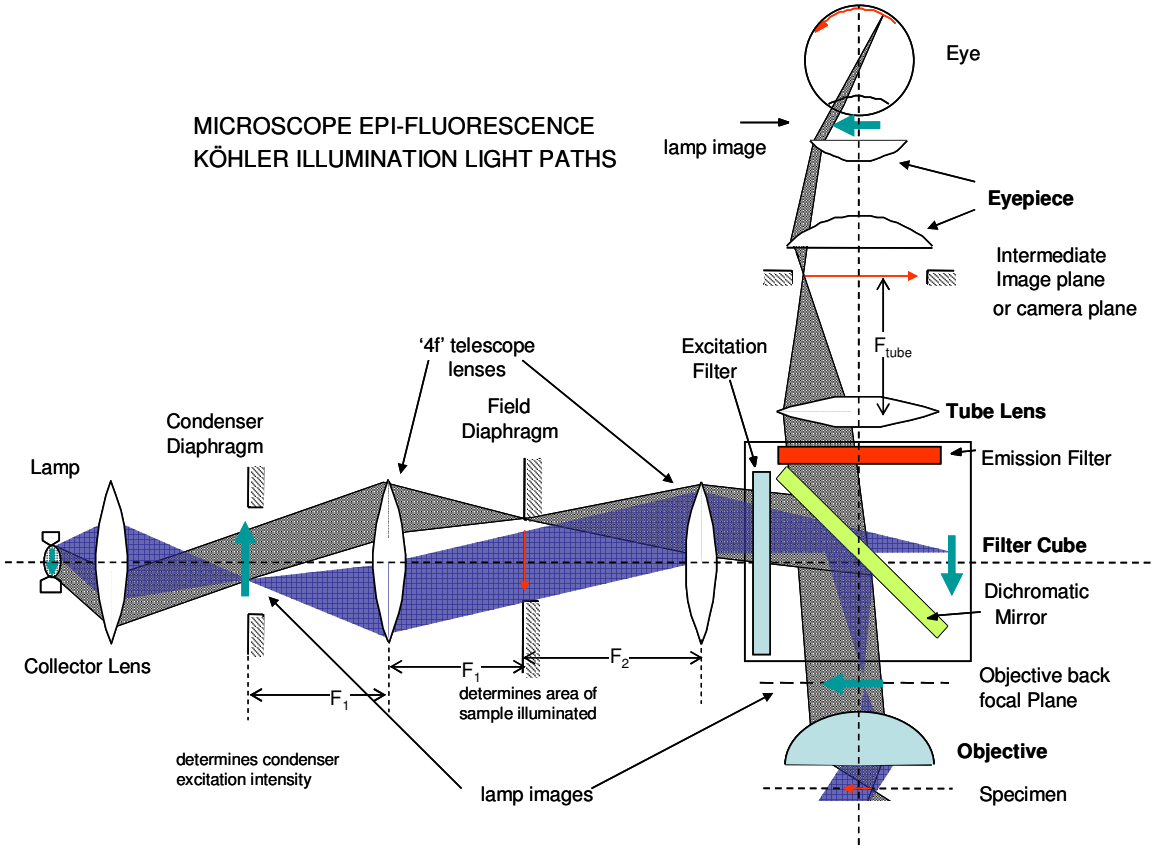


Figure 1: Optical paths in fluorescence epi-illumination, ensuring that a flat illumination field is obtained, with the light source completely defocused at the specimen plane.

The configuration in Figure 1 is applicable to an upright microscope. In the case of an inverted microscope configuration, the arrangement shown in Figure 2 is used: everything is 'upside down'

and the output is deviated so as to be in the horizontal plane. The eyepiece is removed and a camera sensor is placed in the tube lens image plane.

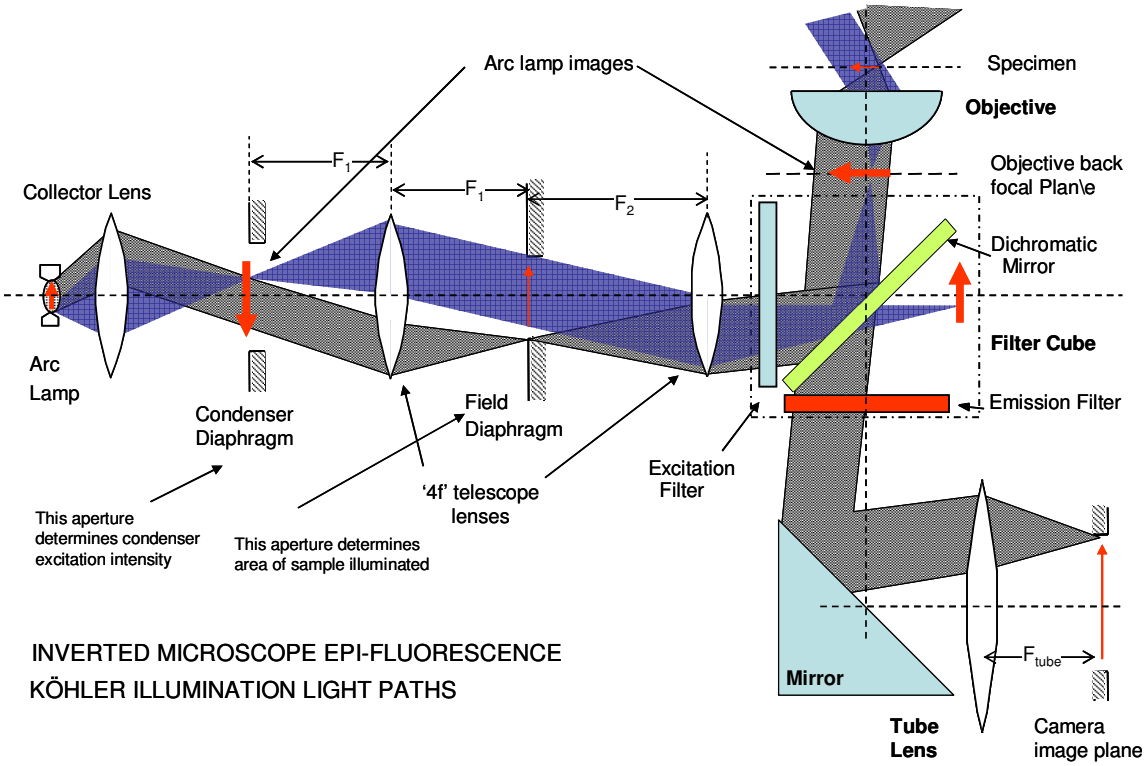


Figure 2: Optical paths in fluorescence inverted epi-illumination.

This configuration is employed in the system described here, with one modification. These days, high pressure arc lamps (mercury or xenon) are slowly being replaced by more complex (but much more stable) illumination sources using metal halide lamps which have a significantly longer lifetime. Such sources are often coupled to the microscope with a liquid light guide the output of which is collimated. We also use such a source, the Prior Lumen 200 (http://www.prior.com/productinfo_illumination_lumen.html), shown in Figure 3. Although the output light guide (typically 3 mm diameter) of such a source could in fact be placed at the condenser diaphragm position in Figure 2, we use an additional lens so as to be able to reduce the source diameter and make it compatible with a 25 mm aperture shutter.

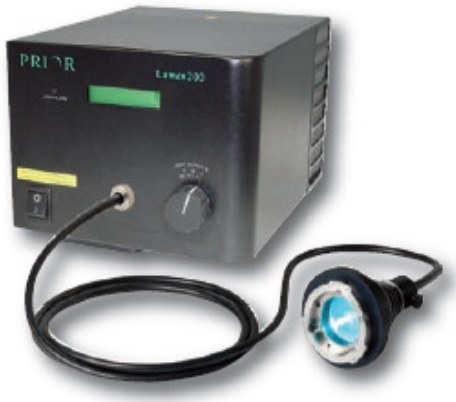


Figure 3: The Lumen 200 light guide coupled metal halide light source

2. The microscope base

It is crucial to ensure that the microscope base is sturdy, free of vibration and as rigid as possible. In commercial microscopes, this is achieved by using a metal cast housing, usually made of aluminium. We start the description of our system with the microscope base and work backwards from this (towards the image plane) and forwards (towards the objective). In our case, we build the microscope directly onto an optical table using rigid spacers coupled to Thorlabs (http://www.thorlabs.com/) cage system components. We start off with two aluminium blocks which couple an LC6W '60 mm' cube to the optical table. This cube houses a kinematically adjusted 40 mm corner mirror (Comar 40 RX 03, http://www.comaroptics.com). Mounting rods (Thorlabs ER8) are also mounted in these blocks, go through other optical assemblies and terminate in the focus assembly, described later, as shown in Figure 4.

The mirror is mounted on a plate (shown in green in Figure 5) which has a machined lip on it to ensure that the corner mirror is mounted orthogonally. Three spring-loaded adjusters provide tip-tilt and translation adjustment to allow the input and output beams to be made are coaxial with the cube apertures and normal to the cube top and side faces.

This basic block is used to mount a fluorescence cube assembly, described elsewhere (please see document "A motorised fluorescence cube linear positioner" on our web site).

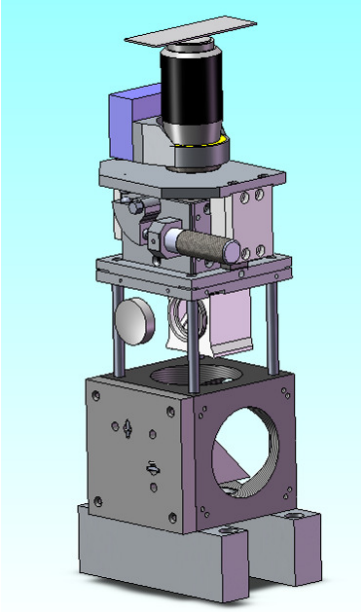


Figure 4: The basic assembly of the inverted microscope: two blocks attach a 'cube' (60 mm) which houses the image turning mirror. A fluorescence cube assembly is placed on top of this and the image-forming optics are completed with a focus assembly and objective.

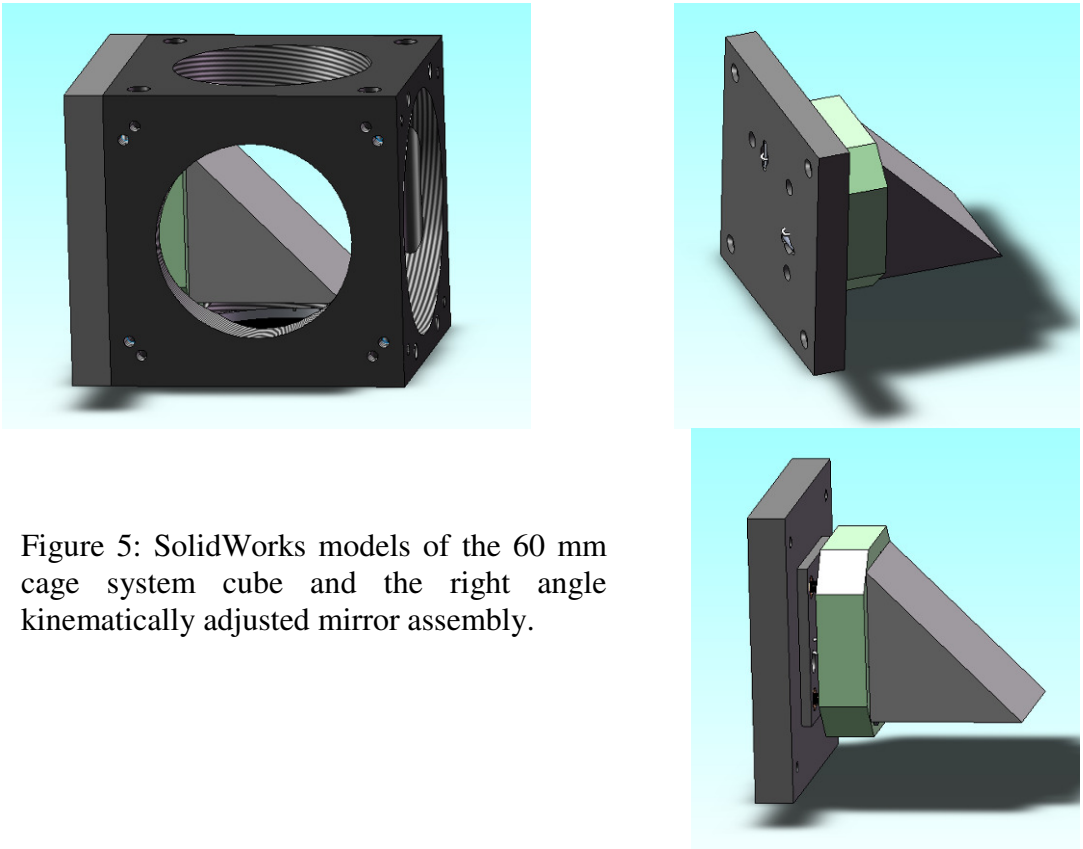


Figure 5: SolidWorks models of the 60 mm cage system cube and the right angle kinematically adjusted mirror assembly.

The output of this 'cube' is coupled to the microscope tube lens. A Nikon 200 mm focal length tube lens can be readily obtained from Edmund Optics (part NT58-520, http://www.edmundoptics.com, at reasonable cost (£169 at time of writing).
This is shown in Figure 6 and is mounted in a 60 mm plate (Thorlabs LCP03/M) held by mounting rods, as shown in Figure 7.

Appropriate tubes could be used to couple a 'C'mount camera such that the camera's flange is placed 133.7 mm away from the tube lens' output flange (the sensor to camera flange distance on 'C' mount camera is 17.53 ± 0.1 mm (ISO 10935:1996 / BS 7012-9:1997). In our embodiment, we place an optical switch just before the camera so as to be able to guide the tube lens output to one of several output ports (please see "A four position motorised optical path selector" on our web site) as shown in Figure 7. The same tube lens-camera flange distance is ensured using tubes of appropriate length.

We note that the tube lens-to-objective distance is specified to be maintained to less than 200 mm. This distance limitation is so as to prevent vignetting of the output field, nominally around 22 mm for the combination of Nikon CF160 and tube lens combination. However, when the output field is somewhat smaller (e.g. a 2/3" CCD's diagonal distance is 11 mm), the maximal 200 mm distance requirement can be somewhat relaxed. Indeed, should this arrangement be used in a laser scanning system, the tube lens distance should be set so as to ensure a stationary excitation beam at the objective's rear focal plane.

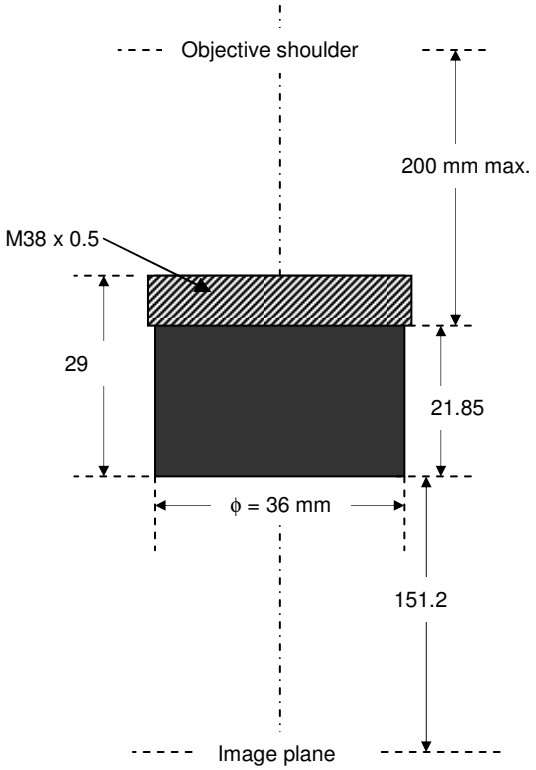


Figure 6: the Nikon 200 mm tube lens. The image plane must be maintained at 151.2 mm form the tube lens rear shoulder and the objective should be placed at a distance not significantly exceeding 200 mm from the lens M38 threaded plane.

3. Objective mounting

The objective focusing assembly is arguably one of the most critical and delicate parts of any microscope. Commercial systems employ a sophisticated mechanical arrangement which can drive the objective in a 'true' vertical path with nanometer resolution and it is common to find multiple gearing arrangements use to provide both coarse and fine focus adjustments. Such assemblies are necessarily large and heavy in order to ensure stability and precision of movement. One of the consequences of this is that fast focus changes are not readily achieved and, when motorised systems are envisaged, such speed limitations can often be troublesome.

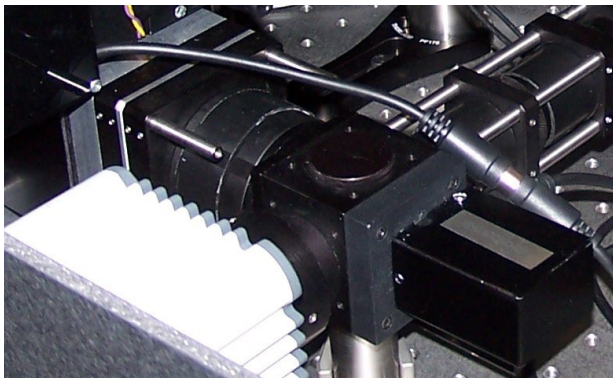


Figure 7: Mounting of the tube lens.

In our arrangement, we also use two systems to provide coarse and fine movements. However, the coarse movement is intended to be just that: it is not intended to provide continuous focusing, but rather to drive the objective to a specific sample-dependent height so as to allow a very fine focusing operation to be performed. The coarse movement uses a dc motor while the fine movement uses a piezo-electric drive system. These systems are stacked on top of each other, as shown in Figures 8 and 9, with the coarse movement arranged to be placed around a C6W Thorlabs cube.

The coarse microscope objective movement is based on a 90 degree bellcrank arrangement (http://www.daerospace.com/MechanicalSystems/Bellcrank.php) similar to that used in a Comar type 50 XT 45 elevation stage (this is the unit that gave us the idea!), but the bearings are replaced with a precision ballslides. Two linear ballslides, Deltron type N-1AC (http://www.deltron.com/) are used on wither side of a the C6W cube, holding a platform into which the piezo drive assembly is screwed.

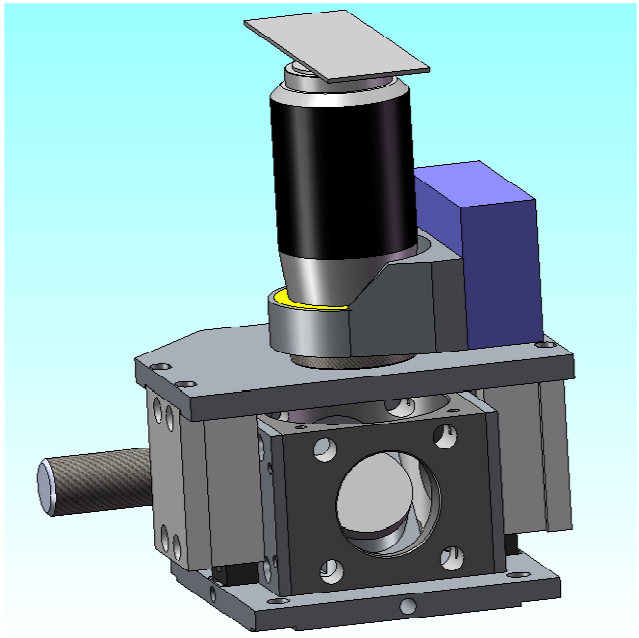
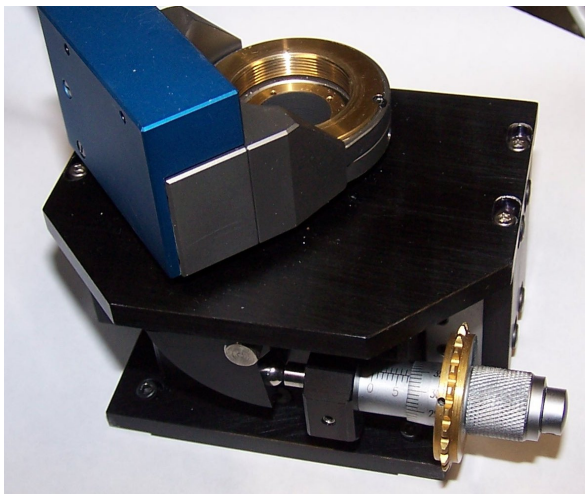


Figure 8: SolidWorks model of the inverted microscope's focusing system.

The coarse drive system can be operated with a micrometer or, through a toothed belt and drive wheel shown in Figure 9, by using a DC motor drive. An appropriate drive system is described elsewhere (please see "DC motor-encoder position servo controller" on our web site.

The piezo objective drive assembly is manufactured by Piezo Jena and available as MIPOS 500 SG-UD with O-360-01 adaptor (take care, the UD suffix is important!) available from: http://www.piezojena.com/en/site/site/z-axis-lens-positioning__196/. The associated controller is a Eurocard-mounted version of their 12V40 controller, although other stand-alone controllers would be equally suitable.

Although the open-loop control range for this type of drive is +/- 250 µm, the closed-loop control range does not exceed +/- 200 µm; this is more than sufficient to take care of sample height variations once the coarse focus is set.



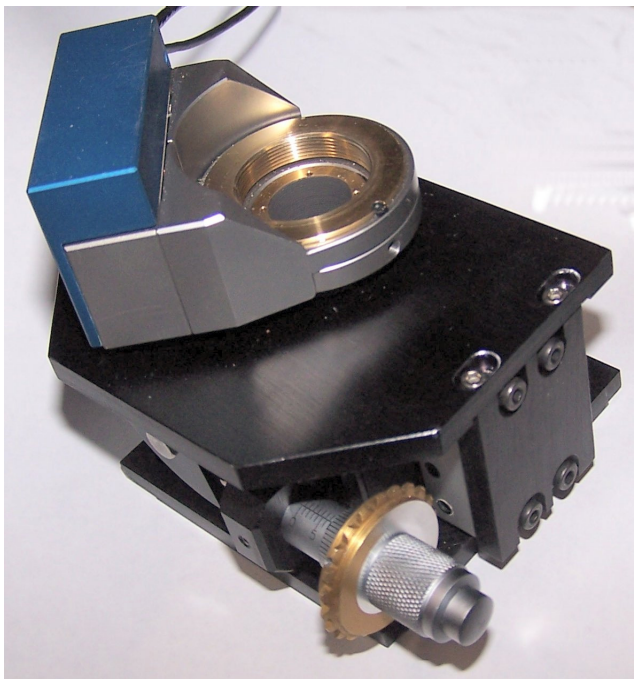


Figure 9: The inverted microscope's focusing system showing details of the micrometer-driven paddle drive (top) and the vertical linear stages (right).

The C6W cube around the coarse drive serves a secondary, but nevertheless very useful purpose: a sampling power meter can be inserted into one of the side ports. Such a power meter is described in the note "Metering systems to determine excitation intensities in fluorescence microscopy" available on our web site.

4. Excitation path

The fluorescence excitation optical train starts with the metal halide lamp, light-guide coupled (light guide diameter 3 mm) to a ~50 mm focal length collimating lens housed within a Nikon bayonet mount attachment. This is camped into a female bayonet mount attached to a Thorlabs LCP03/M plate, as shown in Figure 10. Although the female bayonet mounts are available from Nikon, lately delivery times have become significant and other users may prefer to mount the lens assembly directly within the LCP03/M plate.

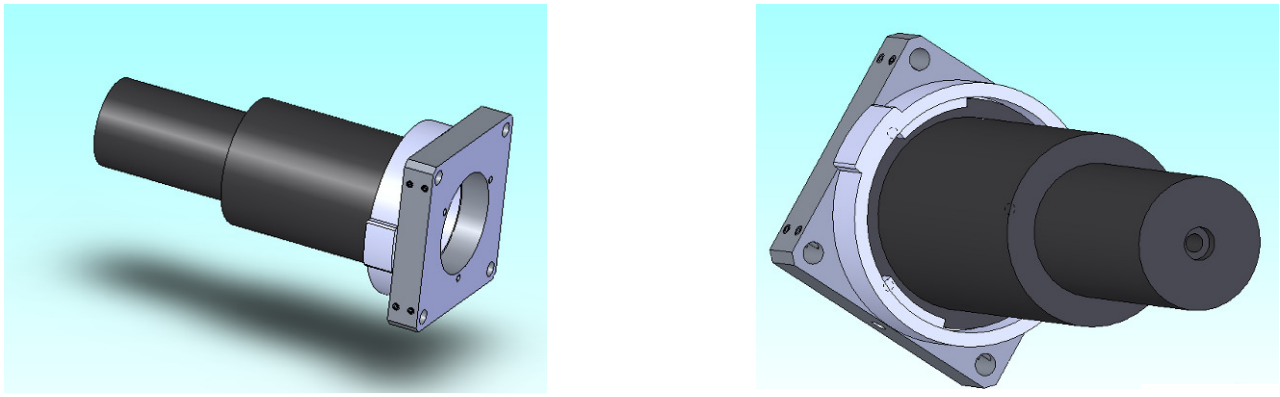


Figure 10. SolidWorks model of the light guide collimating lens assembly.

The spectral output from the lamp/light guide assembly is not particularly flat but is quite adequate for the majority of visible (and NIR) fluorophores as shown in Figure 11.

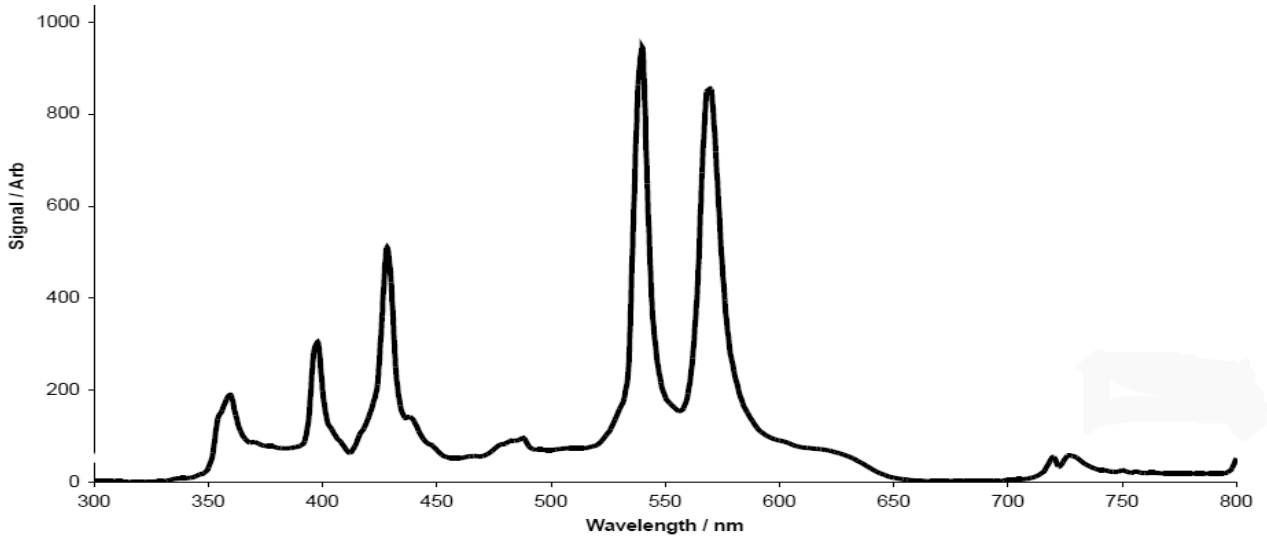
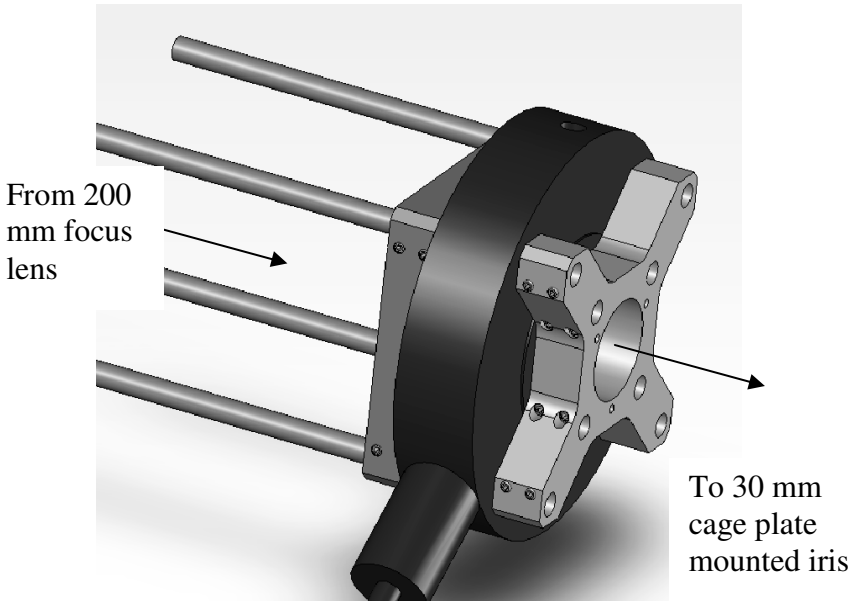


Figure 11: Lumen 200 output spectrum

A 50 mm diameter 200 mm focal length lens, mounted in a Thorlabs LCP01/M 60 mm cageplate (a LCP01T/M would have been better, but was not available at time of system development) is used to provide a 12 mm image of the light guide on the condenser diaphragm aperture (a Thorlabs

SM1D12D ring adjustable iris. Just before this aperture, we place a fast excitation shutter (please see the note "Uniblitz optical shutter driver" for detailed explanations. The shutter assembly (figure 12) also provides conversion between the 60 mm and 30 mm Thorlabs cage mount systems, since at this point, the diameter of the light patch emerging from the 200 mm lens converges to some 20 mm, allowing it to pass through the 25 mm shutter on its way to the condenser diaphragm aperture. In many instances, the actual iris can be dispensed with altogether, but it is nevertheless useful to be able to restrict the fluorescence excitation intensity.

Figure 12: The Uniblitz shutter mounting assembly which also converts between 60 mm and 30 mm cage plate rod-mounting systems



The 'shuttered' excitation light then passes through the first of the two '4f' telescope lenses described in Figure 2, is folded, passes through a field aperture, is folded again and enters the second of the two '4f' telescope lenses. This is shown in Figure 13 and perhaps more clearly in Figures 14 and 15.

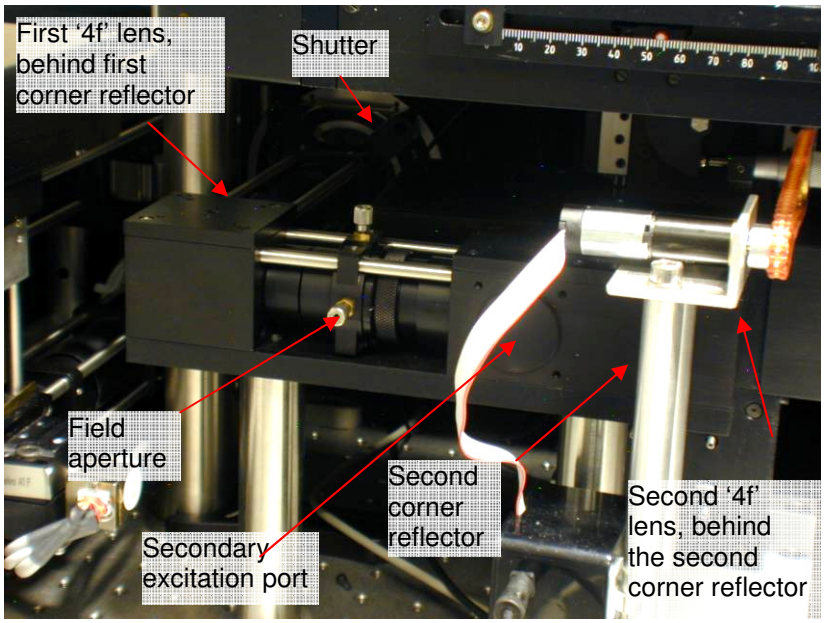


Figure 13: The optical path following the shutter. Excitation light passes through the first of the '4f' lenses before turning through 90 degrees Reflection is provided by an adjustable corner reflector prism mounted in an excitation block, which also houses the field aperture, mounted on a centering stage, a secondary excitation port and a further turning prism. The second '4f' lens is placed just after this and just before the fluorescence cube assembly.

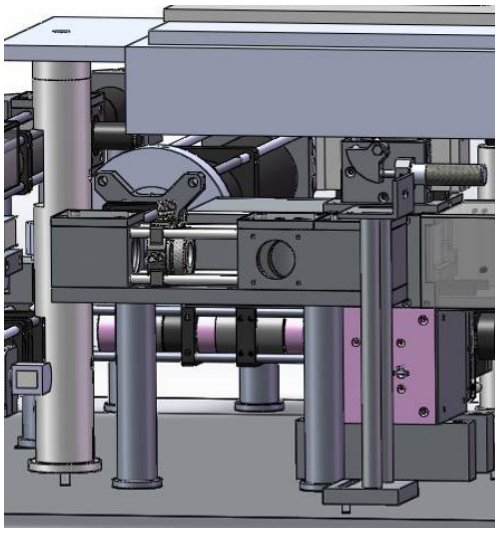


Figure 14: The optical excitation path following the shutter, view from side of instrument.

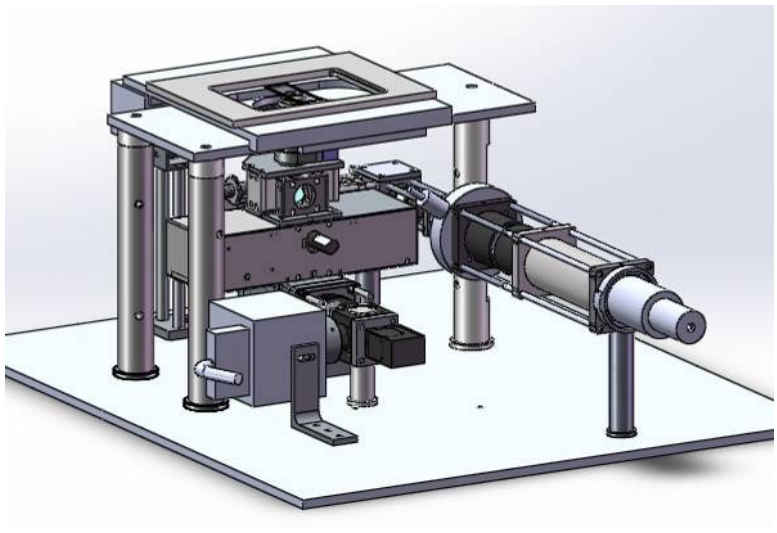


Figure 15: The optical excitation path following the shutter, view from shutter side.

The full optical path diagram is shown in Figure 16. All of the optical components can be readily obtained for the usual suppliers and these are listed overleaf, in Table 1.

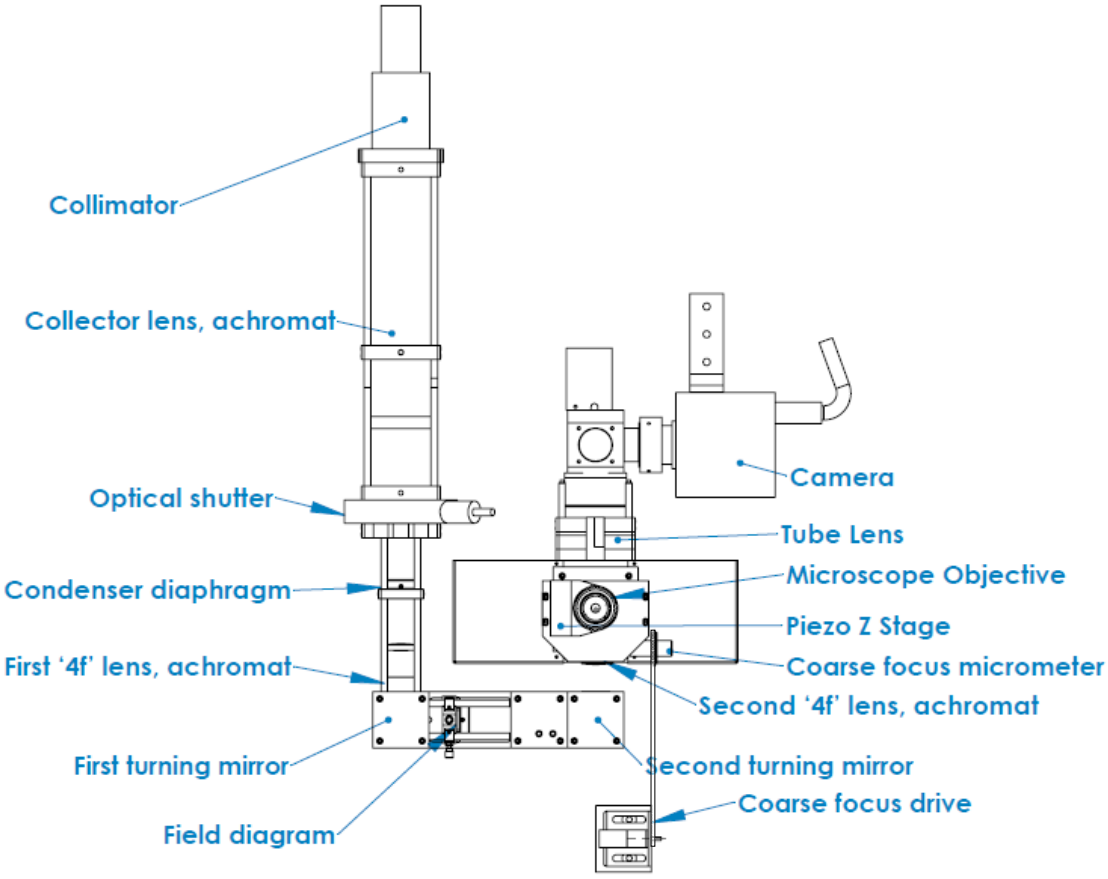


Figure 16: Plan view of the excitation optical path.

Table 1: Fluorescence excitation optical components

Component	Description	Part number	Supplier
Collector lens, achromat	ϕ = 50.8 mm 200 mm fl	AC508-200-A1	Thorlabs
Optical shutter	25 mm fast shutter	VS25S2ZM1	Uniblitz
Condenser diaphragm	Ring activated 12.5 mm	SM1D12D	Thorlabs
First '4f' lens, achromat	ϕ = 25.4 mm 100 mm fl	AC254-100-A1	Thorlabs
First turning mirror	25 x 25 x 25 mm	MRA25-F01	Thorlabs
Field diagram	Ring activated 12.5 mm	SM1D12D	Thorlabs
Second turning mirror	25 x 25 x 25 mm	MRA25-F01	Thorlabs
Second '4f' lens, achromat	ϕ = 25.4 mm 150 mm fl	AC254-150-A1	Thorlabs

5. System images

Real-life and model images of the complete microscope system are presented on subsequent pages in Figures 17-20. They are essentially self-explanatory. Details of the other subsystems can be found on complementary technical notes, which should be read in conjunction with this note.

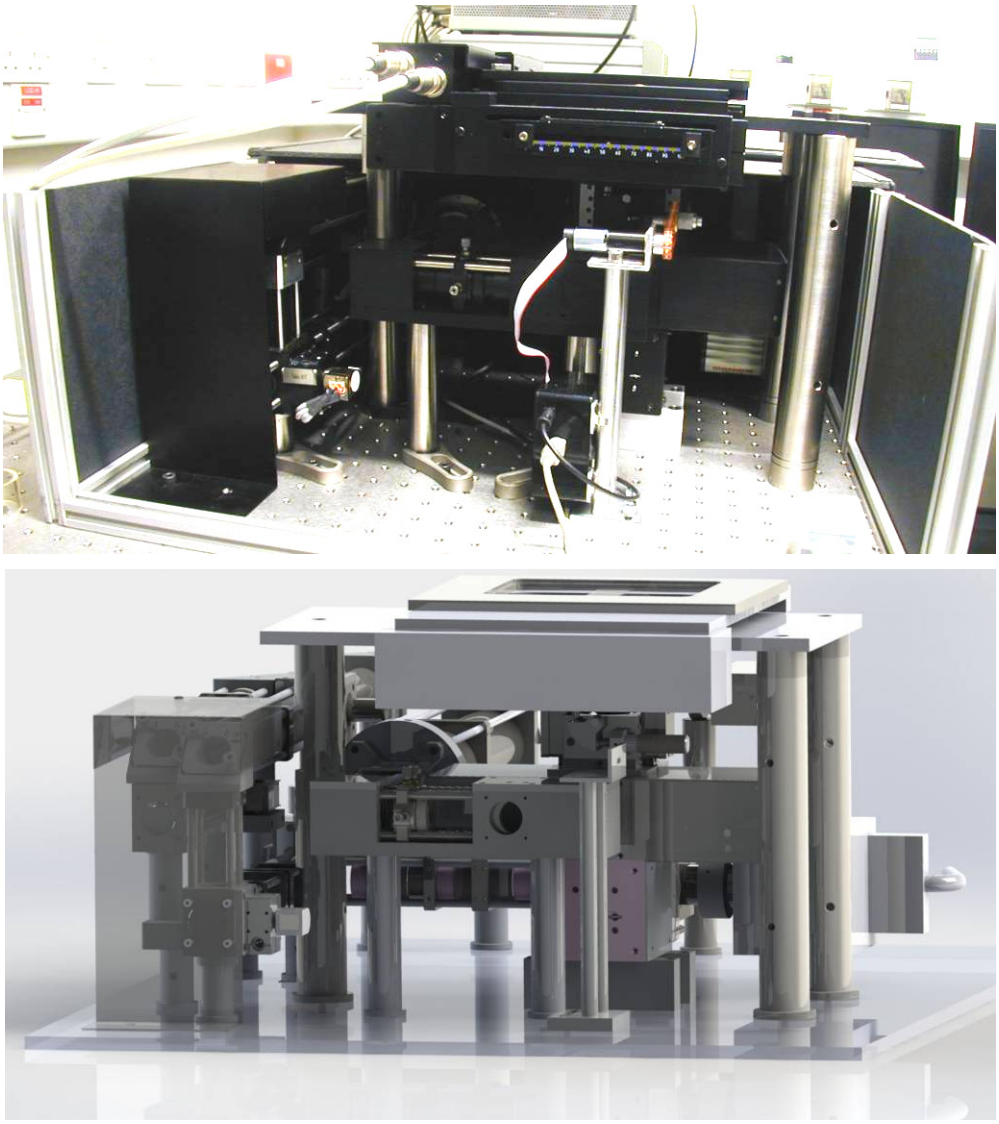
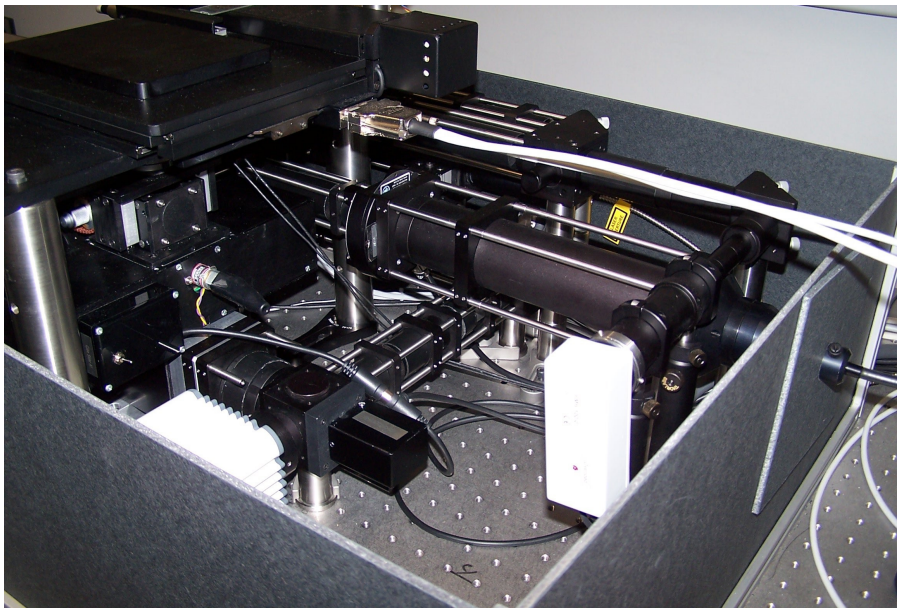
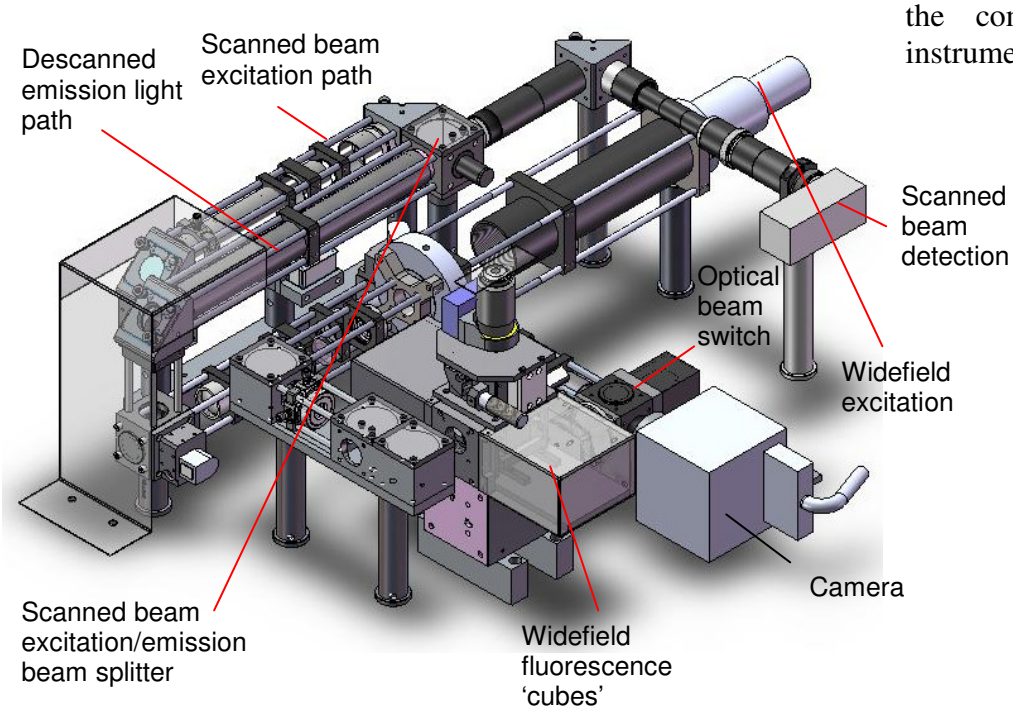


Figure 17: The instrument from the side. To the left of the shutter, behind a protective cover, are placed optics associated with laser beam scanning, described separately.





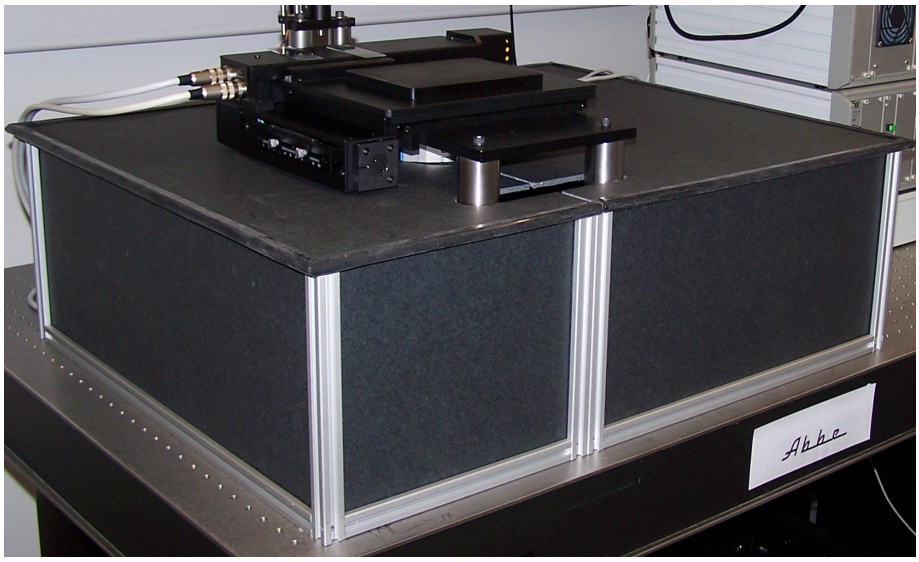
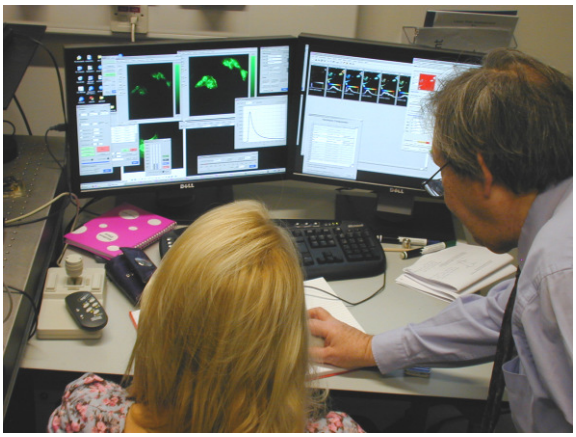
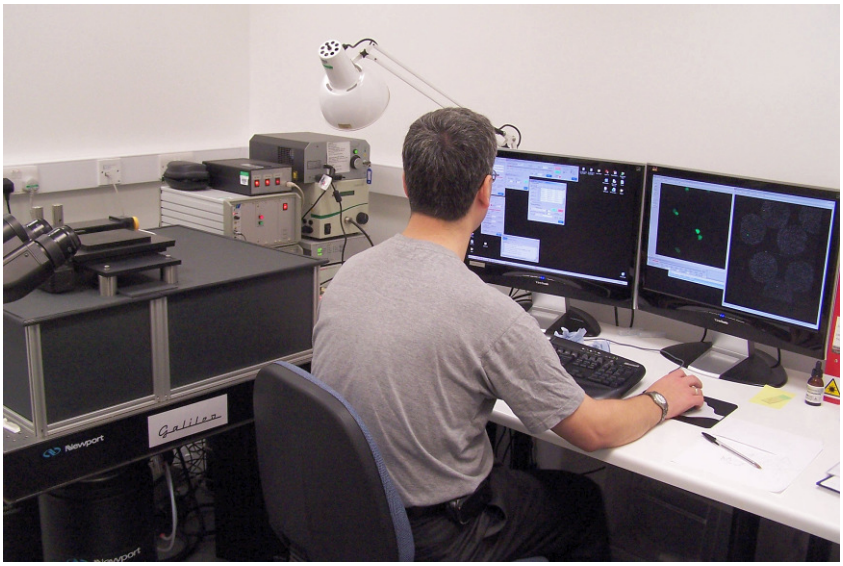


Figure 18: The completed instrument, showing the optical path switch (upper bottom left) which couples the output of the tube lens to the camera or two a laser scanning path, described separately, and terminating in a photomultiplier detector (white box in upper left side).

The middle panel shows a bird's eye view of a model of the instrument.

The bottom panel shows the completed, enclosed instrument.





The method described here was originally conceived in 2004; this note was initially prepared in 2008 and updated during September and October 2011. Initially three versions of the device shown were constructed: two are in operation at King's College London (one of which is shown in the bottom panel), and one at the Gray Institute in Oxford (upper panels). Another similar, but simpler version was constructed for collaborative work at the Health Protection Agency and a significantly more complex device was installed at the University of Surrey, applied to work associated with hadron microbeams. Finally a version for application with the Gray Institute's linear accelerator facility was installed during 2011.

B Vojnovic, IDC Tullis and PR Barber contributed to this note and IDC Tullis performed most of the instrument assembly. The mechanical items were fabricated by John Prentice and Gerald Shortland. Detailed drawings of the various mechanical parts are available on request for non-commercial users who wish to copy this system.

We acknowledge the financial support of Cancer Research UK, the MRC and EPSRC.