Make your own automated microscopy system

B. Vojnovic and P. Barber
Grey Cancer Institute

The obvious question is: 'Why bother?' After all, there are numerous microscopy and image processing systems on the market capable of excellent general-purpose performance. And therein lies the answer: they are necessarily designed for general use and perhaps not always optimised for a particular task. While most of them can be tailored for a specific application, very often a significant amount of software development time and expertise is required and some suppliers are not too keen to divulge their 'secrets'. Furthermore, commercial systems are limited to controlling a standard set of hardware and any 'peculiar' additions tend to be frowned upon. Finally, you may have access to existing microscope bodies, perhaps not the latest devices available, and, as most scientists are all too well aware, funding agencies have an increasing tendency to demand 'preliminary data' or 'proof of principle' before considering a grant application favourably. Another reason for developing such systems is associated with the occasional need to couple them to other 'automated' systems; in our case, micro-irradiation systems feature high on the list. But of course the real reason is much simpler: 'It is both interesting and fun to do'. Although we would never admit to this, there is something particularly satisfying in developing a device making all kinds of whirring noises all by itself and coming out with a brightly coloured image at the end of the process!

More seriously though, here we describe some our experiences. The most important decision is the type of camera to use and the optics used to couple it to the microscope. Most microscope suppliers offer coupling optics for their 'scopes, but a useful source of custom optics, or when older microscopes are used is MicroCam Ltd (see addresses at end of document). In general we favour using no coupling optics at all and using the largest possible imager to cover as much of the available field of view from the microscope. In practice this means restricting the FOV to 11 mm with a 2/3" camera. In general, the greater the demagnification, the poorer the image quality; but this is very much a generalisation and it is useful to check performance with the actual camera/optics used. Zoom coupling optics are certainly useful, albeit expensive, and there needs to be some good reason why you would prefer not to use the most appropriate objective for the required magnification. If you do use a zoom system, use a device with defined 'click' stops rather than a continuously variable one: it will be much easier to calibrate distances in software with defined positions.

The camera

As far as cameras are concerned, the choice is extensive but really the choices to make are: video rate or digital interface, monochrome or colour, then take a close look at your available funds. Video rate (CCIR) cameras are relatively cheap but you need to include the cost of a frame grabber in the overall system cost. Video rate cameras provide an interlaced output and are thus often not well suited to observe dynamic events. Colour cameras come in basically two forms: single chip or three or more chips. The single-chip devices have an array of red, green and blue filters on adjacent pixels and the pixel resolution provided is thus lower than that of 3-chip devices, which split the input light through dichroic filters onto individual CCD chips. Resolution is thus not impaired, but watch out for colour shading problems, especially if high demagnifications are used. Over the last few years, we have used the following colour cameras:

JVC KYF 55   3 x ⅓" CCDs, video rate
JVC KYF 58   3 x ⅓" CCDs, video rate
JVC KYF 70   3 x ½" CCDs, video rate and SCSI bus
JVC KYF 75   3 x ½" CCDs, IEEE1394 ('Fire Wire') bus

None of these are particularly suited for ultra-sensitive fluorescence work but are useful for general histology or similar non-photon-limited microscopy.

When it comes to low-cost monochrome cameras, the Cohu 4900 range of cameras is hard to beat. With these many operating modes are readily preset, full frame integration is readily achieved and they can be easily modified to provide an external gain control input (by overriding an internal, gain-defining preset potentiometer, which delivers 0 to +5V to internal gain-control circuitry. A cooled version of the 4900 is available and is excellent for very low light level work. A good contact for the Cohu series of devices is BRSL (addresses at end of document).

An interesting high sensitivity device has recently become available, Cohu 2700 series, which has a very wide dynamic range of operation and a sensitivity comparable to that provided by 1st generation intensified cameras, but at a fraction of the cost. These seem to be excellent for following dynamic fluorescence events, e.g. in intravital microscopy, but as supplied do not allow external control of gain; we are currently investigating how to modify them.

All of these devices provide an adequate, but limited pixel resolution of 768 x 512 at best, and need to be used with a frame grabber. For more serious work, mega pixel cameras are really to be preferred; these typically provide 1344 x 1024 pixels and demand some form of digital interface. Our experience is restricted to SCSI interfaces (slow and not recommended, but that was all that was available some years ago), to LVDS systems (perfectly acceptable but require relatively expensive and often specific cards in the host PC) and to IEEE1394 ('Fire Wire') bus devices. Of all of these, the last is most convenient to implement and get working quickly. Although the transfer speed can be restrictive (around 10 fps is typical), the ease of use and the attraction of an interface card costing a few tens of pounds makes this a current 'favourite'. For the future, the CamLink standard is the one to go for, but the range of devices available for this standard is still somewhat restricted.

We have often used the Hamamatsu ORCA series of monochrome cameras and find these relatively easy to interface. The 2/3", back-thinned, cooled versions are adequate for all but the most demanding low-level fluorescence imaging. The acquisition phase of these full-frame (i.e. non-interlaced) devices can be triggered with an external TTL signal and they are thus well suited for use with excitation shutters.

The excitation shutter

For any serious fluorescence work, a fast-acting shutter in the excitation path is definitely advisable. There are numerous types available, but we had been familiar with the Uniblitz range of devices from Vincent Associates, and have successfully used these in numerous applications that they were never intended for, such as shuttering low energy x-ray beams, proton beams etc.! These shutters can be driven very fast (on-off transition times of 2-3 ms), and drivers are available for this purpose, but we have found that a gentler (5-8 ms energisation time) improves reliability considerably. The way that fast energisation is obtained is by applying a high (30-80V) amplitude voltage pulse to the operating solenoid and gradually decrease the voltage over a few milliseconds to provide an appropriate 'holding' current. This is essentially achieved by discharging a capacitor through the coil and the operating conditions are defined by the charging voltage and the capacitor value. A simple, reliable circuit is presented in the section 'Useful circuits'.

There are numerous cased and uncased versions of these shutters available from BFI Optilas Ltd. Various adaptors are also available from the same source to fit most microscopes. The most suitable devices for most systems are the 25 mm diameter range of shutters and it is recommended that units fitted with a feedback mechanism are used: it is then much simpler for other hardware/software to 'know' the state of the shutter. We are most familiar with the Nikon range of microscopes but are too

mean to pay what seem to be high amounts for very simple mechanical adaptors. Having access to an excellent mechanical workshop, we can afford to be mean, since it only takes a short time to machine suitable adaptors and couple them to bayonet-type adaptors available from Nikon (BMS and BFS adaptors). The arrangement is shown in Figure 1. Note that convection cooling slots are incorporated between lamp and shutter to aid heat dissipation, particularly when the shutter is 'closed'.
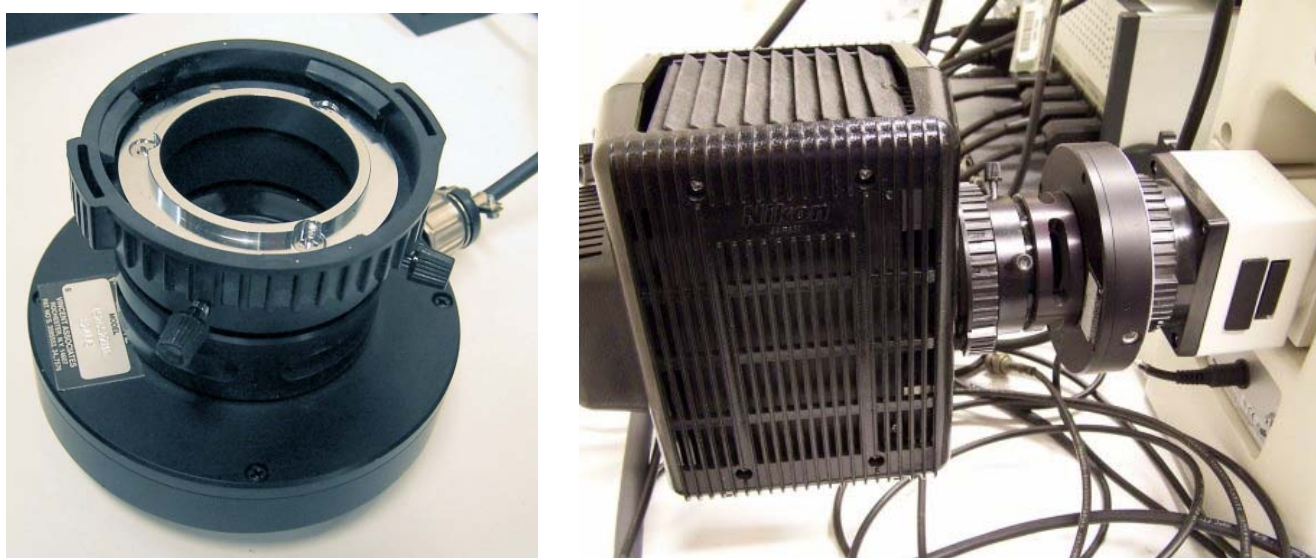

Figure 1. View of shutter assembly for Nikon microscopes

The stage

A motorised microscope stage is not only very useful but also essential when any form of automation is contemplated. We have tended to utilise the Marzhauser range of stages, with a variety of controllers. Most of their stages are stepper-motor driven devices (actually operated in micro-stepping mode) with step resolutions of 250 nm or better, using a 1 mm pitch leadscrew. It is worthwhile remembering that the (micro)step resolution has nothing to do with absolute accuracy: the latter is defined by the tolerances associated with the leadscrew and the precision of the bearings. The bearings ultimately dictate the achievable accuracy, which really should not be expected to be much better than +/- 2 µm, primarily due to the fact that the drive is to the side of the 'required' position which itself is sensed from motor pulses delivered. It is an 'unfortunate' mechanical issue that microscope stages need a 'hole' in their centre – it would be a simple matter otherwise to sense the actual position very precisely indeed, along the axis, where it is required.

It is an inevitable indication of 'progress' that motor controllers have evolved over the years, though a consequence is that improved performance comes at the price of differences in driver software, often requiring major rewriting. It helps however to maintain a similar user interface (GUI) so that operation procedures at least appear comparable. Our 'general-purpose GUI is shown in Figure 2. One of the issues between different controllers is their ability to set independent acceleration and velocity profiles: while this is of negligible consequence for X and Y axes, when a third (Z or focus) axis is added, the latter can be limiting in terms of the achievable performance. It is a good idea to characterise the Z-drive of the microscope before a firm decision is made as to whether a 2-axis or 3-axis controller is purchased.
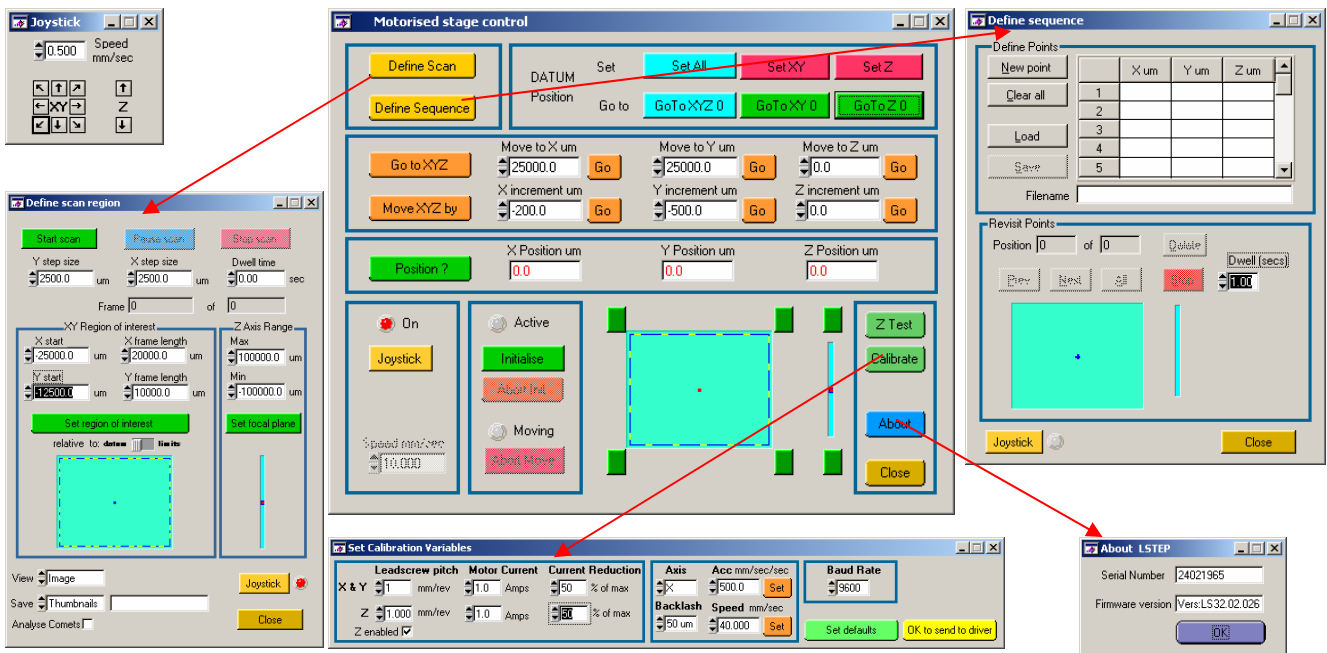
3

Figure 2: Stage control GUI's, which allow control of move to absolute or relative coordinates, the ability to store and recall a sequence of coordinates, to scan the sample in a raster-like fashion and to set stage velocity and acceleration profiles and motor operating conditions. A 'map' of where the stage is at any time (relative to the microscope objective) is available on the main, sequence and scan GUIs; the latter can be usefully called from different program sections.

Another often-limiting factor is the speed of communication between controller and host PC. This almost exclusively (and unfortunately) uses a serial RS232 link, which is not always the fastest means. Additional time delays of a few tens milliseconds soon add up if a long sequence of position-image procedures is required. A particularly annoying feature of all external (i.e. not on a PC card) stage controllers is the fact that there is no hardware (e.g. TTL) output, which indicates that stage motion is complete. The addition of such an output would simplify considerably the integration of controllers with imaging devices: e.g. such an output could be used to trigger a camera acquisition, freeing up the host processor to perform image processing rather than take care of communication with the stage controller. Additional notes on stage control can be found in the notes on 'image mosaics'.

How to 'glue' it all together

Ultimately, the 'glue' that holds all the system together is the software. Nevertheless, there are some distinct advantages in putting together a range of hardware and control systems in a single unit which acts as a central control unit for the different opto-mechanical, electro-mechanical and electro-optic systems. This unit then has a single interface cable to the host computer; in our case we have chosen to use the USB standard for interconnection. Rather than design dedicated interfaces, we have adopted a rather more flexible approach, which is easily extended almost indefinitely and caters for any future, as yet unplanned expansions.

The basic idea behind the approach is to use the I^{2}C bus internally, within the system. This is a two-wire bus using serial data and serial clock lines operating at speeds up to 400 kbits sec^{-1}, originally developed by Philips. The great advantage of the bus is that numerous I/O ports, A/D and D/A converters and other subsystems are readily available at low cost from several semiconductor manufacturers. While it is certainly not the fastest interconnection system available, it is an extremely rugged and mature technology and is more than fast enough for controlling mechanical systems, such as in this application. Useful information can be found at www.semiconductors.philips.com/buses/i2c/. Each device (i.e. chip in our case) has a unique address and devices can be daisy-chained almost indefinitely, within limitations set by interconnecting cable capacitance. In practice, the short distances involved in systems such as the ones described here do not limit performance and additional subsystems can be readily added.

So we are left with the 'problem' of converting between a PC-industry standard bus – USB – and the I^{2}C bus. Our approach is perhaps not the most elegant but it easy to develop and modify; it is shown in Figure 3. We make use of a USB to parallel converter module (DLP USB245M, Future Technology Devices Intl. Ltd) connected to a PIC processor (Microchip PIC16F874), which handles the conversion, as well as providing a conventional RS232 serial port and other dedicated I/O lines.
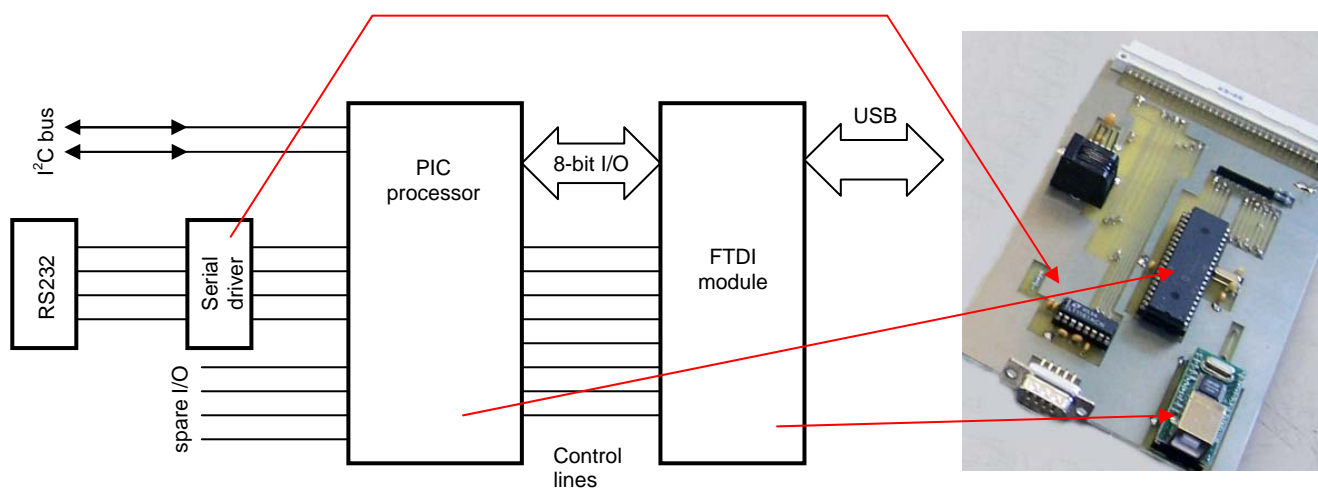

Figure 3: Block diagram of USB interface used in microscopy systems and a practical implementation on a Eurocard format PCB

With this arrangement, it is possible to provide up to sixteen 8 bit parallel I/O ports by connecting PCF8574 and PCF8574A chips to the I^{2}C bus. Multichannel D-A conversion is most easily performed with MAX521 devices (eight 8-bit DACs) or MAX5842 (four 12-bit DACs) while for A-D conversion we find useful to use MAX 127 DAS chips. If the addressing capability of a single I^{2}C bus is reached, multiple local I^{2}C busses can be created by using an I^{2}C-addressable analogue switch, such as the MAX4572; another alternative is the Philips PCA9542. To all intents and purposes, the range of controlled devices is limitless.

Since I/O ports are 'easy' to add, one of them can be put to good use as a multiple AC mains controller to really spoil the user. We use typically 3-4 individually switched mains outputs to provide power to the lamp power supplies, camera(s), stage controller etc. Output switching is accomplished by 5V-operated 16A relays connected to IEC sockets. This not only reduces the number of 13A wall-plug leads but also ensures that arc lamps are never accidentally left switched on – the software takes care of all that at turn-off. It also means that there is only one power switch to turn on for the whole system, i.e. the control electronics.

Of course the real 'glue' is the software. Here it is worthwhile remembering that we need to deal with image acquisition, image processing, signal processing and hardware control within a single environment. For a variety of reasons, we have chosen to base the bulk of the software development within the National Instruments LabWindows CVI™ programming environment. This makes it relatively easy to design, develop and modify both GUIs and the code behind them, particularly when it comes to image and signal processing operations.

It is clearly impossible to describe all the details of the code in a short document as this, but the underlying philosophy is to use a 'modular' approach, where code can be easily interchanged to operate within different systems. As with the example of the stage module described above, it is useful to have standard modules for all commonly used devices as well as functions such as data storage and image display.
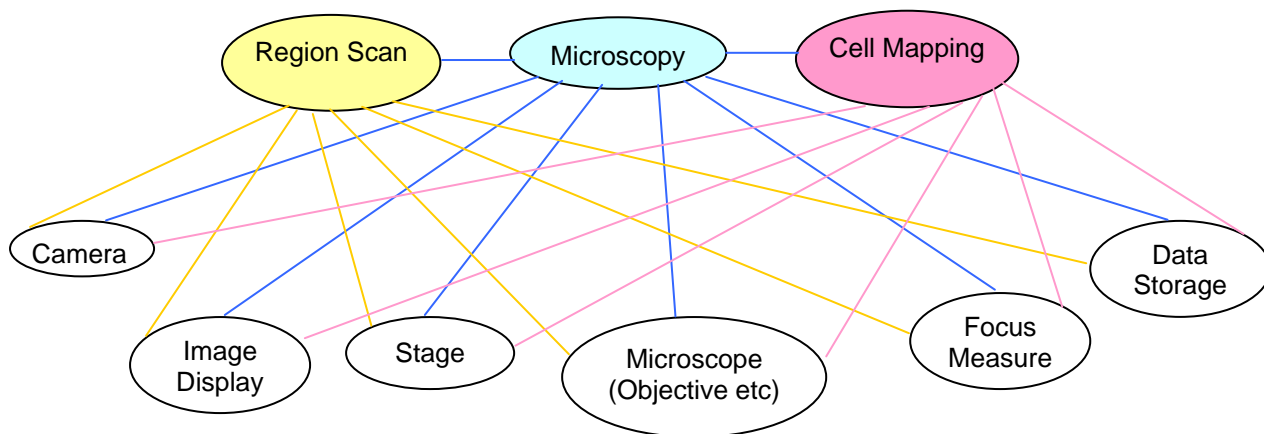

Figure 4: The main software modules required for a basic microscopy system

In Figure 4 above, there is also some necessary interaction between the low level modules, for example every time the **Camera** module acquires an image it must call the **Image Display** module to display it and the **Focus Measure** module to update the focus indicator. Another example is that when 'live' image display is enabled it is desirable to enable the **Stage** joystick and vice versa.

These modules can become quite complex as images can be acquired either as 8 bit or 16 bit monochrome or as 24 bit colour images and stored in a variety of formats with or without overlay information such as a scale indicating object size in microns. It is also necessary to save details of the microscope setup used to acquire the image such as the objective and cube that were in use as well as all the camera settings. A typical camera GUI is shown in Figure 5.
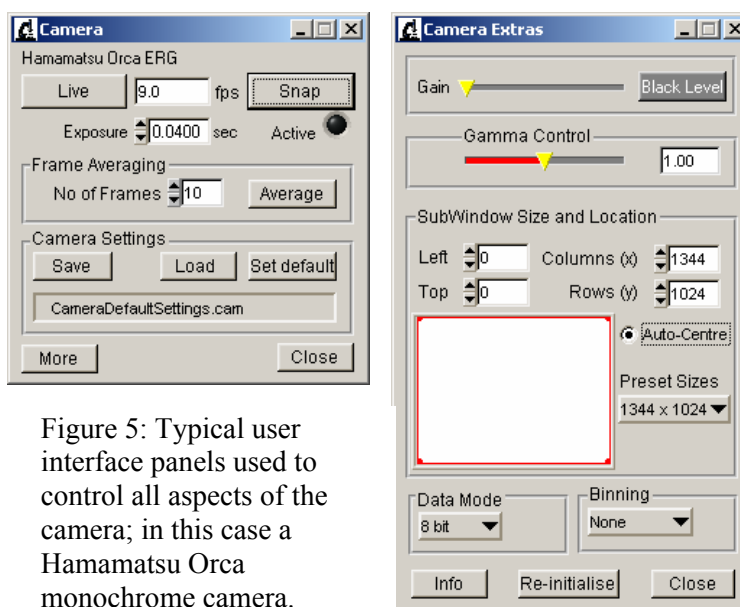

Figure 5: Typical user interface panels used to control all aspects of the camera; in this case a Hamamatsu Orca monochrome camera.

Time-consuming procedures, such as the identification and mapping of all the cells on a slide, must make use of all devices present in the most efficient manner which generally involves multi-threading of functions. The basic application has to make use of several modules simply to acquire and display an image which is in focus. Having acquired the data it can be displayed in a variety of ways. For example a number of false colour look-up-tables have been provided including one that indicates the wavelength of the cube in place during the acquisition in fluorescence mode. Also the focus indicator is active at all times as a useful aid for manual adjustments to the z drive when in 'live' acquisition mode. (The mechanism of the focus indicator is described in the section 'Image Mosaics'.
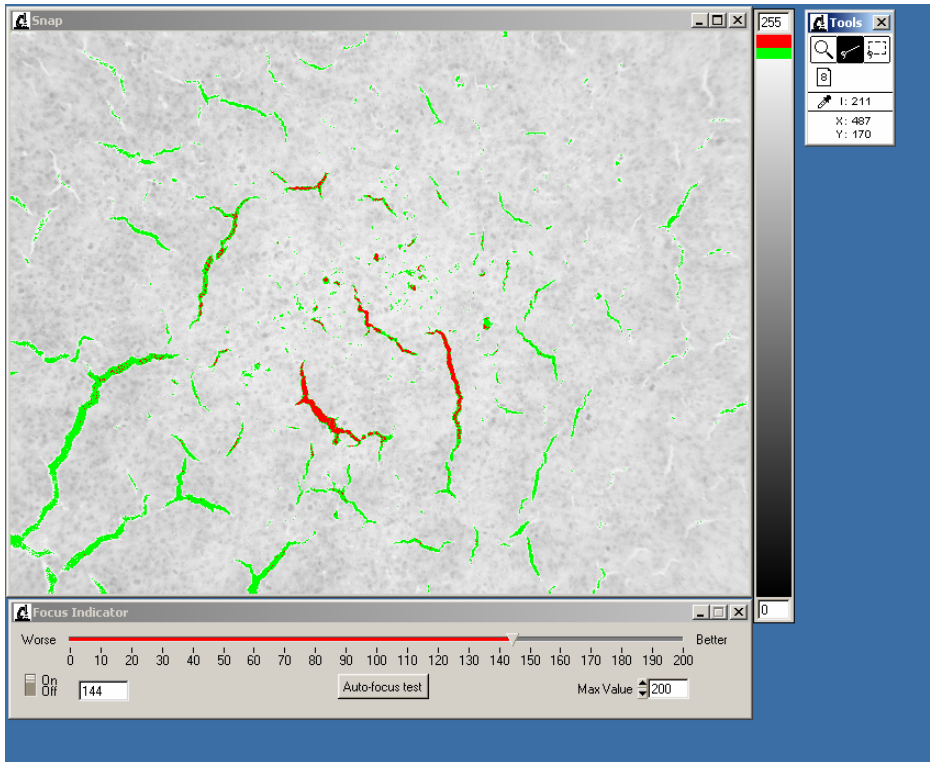
Figure 6: Image displayed using a look-up-table that indicates image overload. The presence of green pixels indicates that the camera dynamic range is about to be exceeded, while red pixels show definite overload.

The **Microscope** module has become much more complex with the arrival of completely automated microscopes like the Nikon TE2000 which enables among other things the objective turret, cube position, condenser, and lamp to be under computer control. On older systems we have added this functionality with our own hardware where possible.

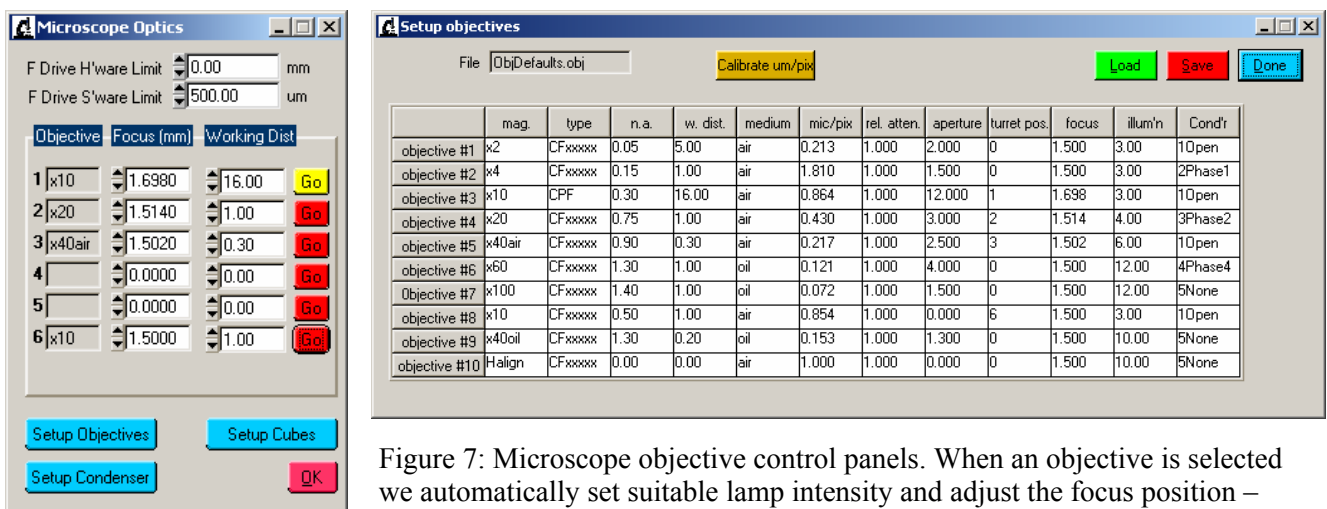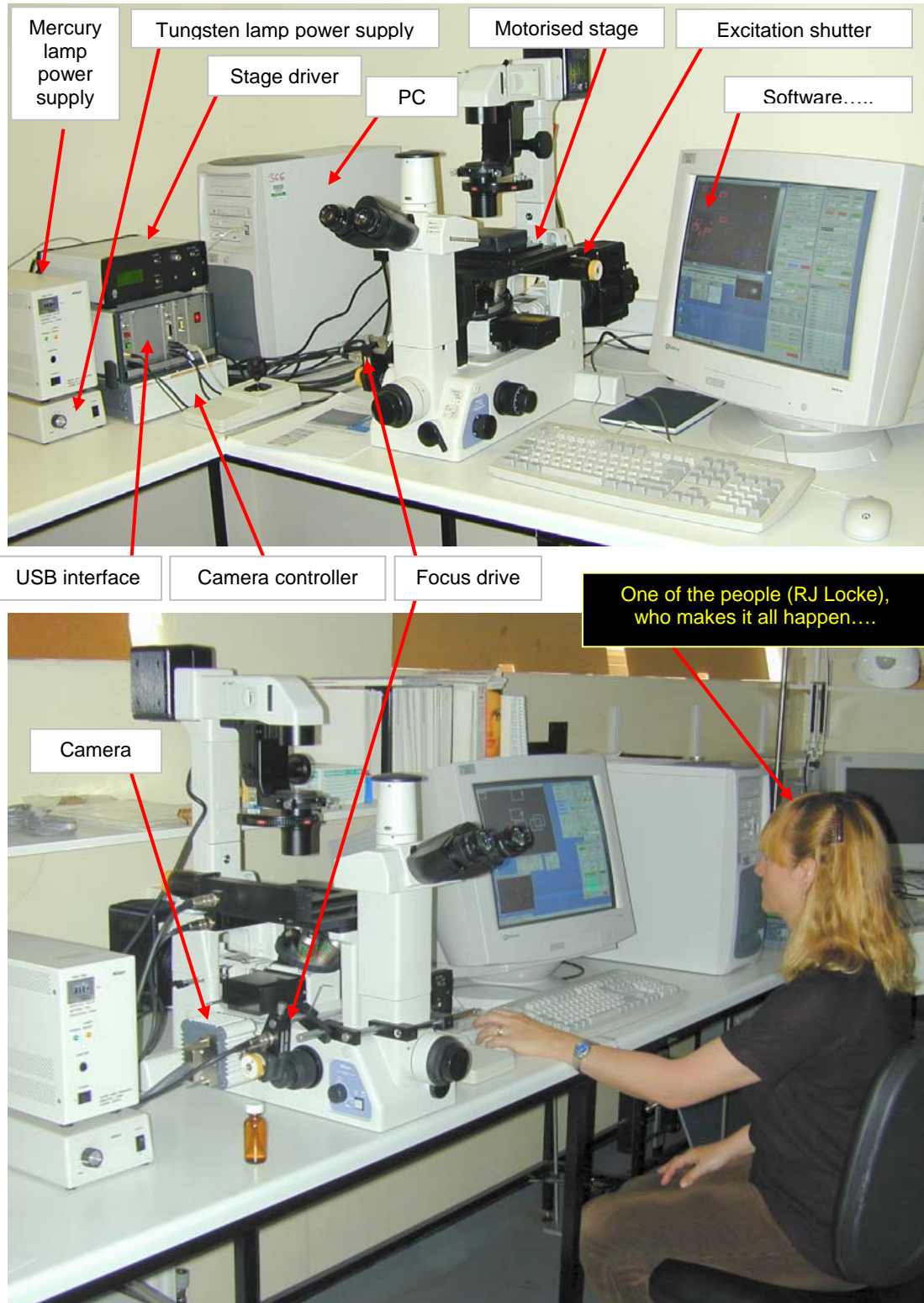|	mag.	type	n.a.	w. dist.	medium	mic/pix	rel. atten.	aperture	turret pos.	focus	illum'n	Cond'r
objective #1	x2	CFxxxxx	0.05	5.00	air	0.213	1.000	2.000	0	1.500	3.00	10pen
objective #2	x4	CFxxxxx	0.15	1.00	air	1.810	1.000	1.500	0	1.500	3.00	2Phase1
objective #3	x10	CPF	0.30	16.00	air	0.864	1.000	12.000	1	1.698	3.00	10pen
objective #4	x20	CFxxxxx	0.75	1.00	air	0.430	1.000	3.000	2	1.514	4.00	3Phase2
objective #5	x40air	CFxxxxx	0.90	0.30	air	0.217	1.000	2.500	3	1.502	6.00	10pen
objective #6	x60	CFxxxxx	1.30	1.00	oil	0.121	1.000	4.000	0	1.500	12.00	4Phase4
Objective #7	x100	CFxxxxx	1.40	1.00	oil	0.072	1.000	1.500	0	1.500	12.00	5None
objective #8	x10	CFxxxxx	0.50	1.00	air	0.854	1.000	0.000	6	1.500	3.00	10pen
objective #9	x40oil	CFxxxxx	1.30	0.20	oil	0.153	1.000	1.300	0	1.500	10.00	5None
objective #10	Halign	CFxxxxx	0.00	0.00	air	1.000	1.000	0.000	0	1.500	10.00	5None

Figure 7: Microscope objective control panels. When an objective is selected we automatically set suitable lamp intensity and adjust the focus position – objectives are not always parfocal. Parameters for all available objectives can be edited and stored by suitably qualified individuals, and we have included a calibration tool to readily determine the microns/pixel ratio.

Some examples of complete systems

Mercury lamp power supply · Tungsten lamp power supply · Stage driver · PC · Motorised stage · Excitation shutter · Software….. · USB interface · Camera controller · Focus drive · Camera · One of the people (RJ Locke), who makes it all happen….

Examples of an early version of an automated system intended to quantify fluorescence images from 'comets'; this is based on a Nikon TE300 microscope coupled to an Hamamatsu ORCA series camera.
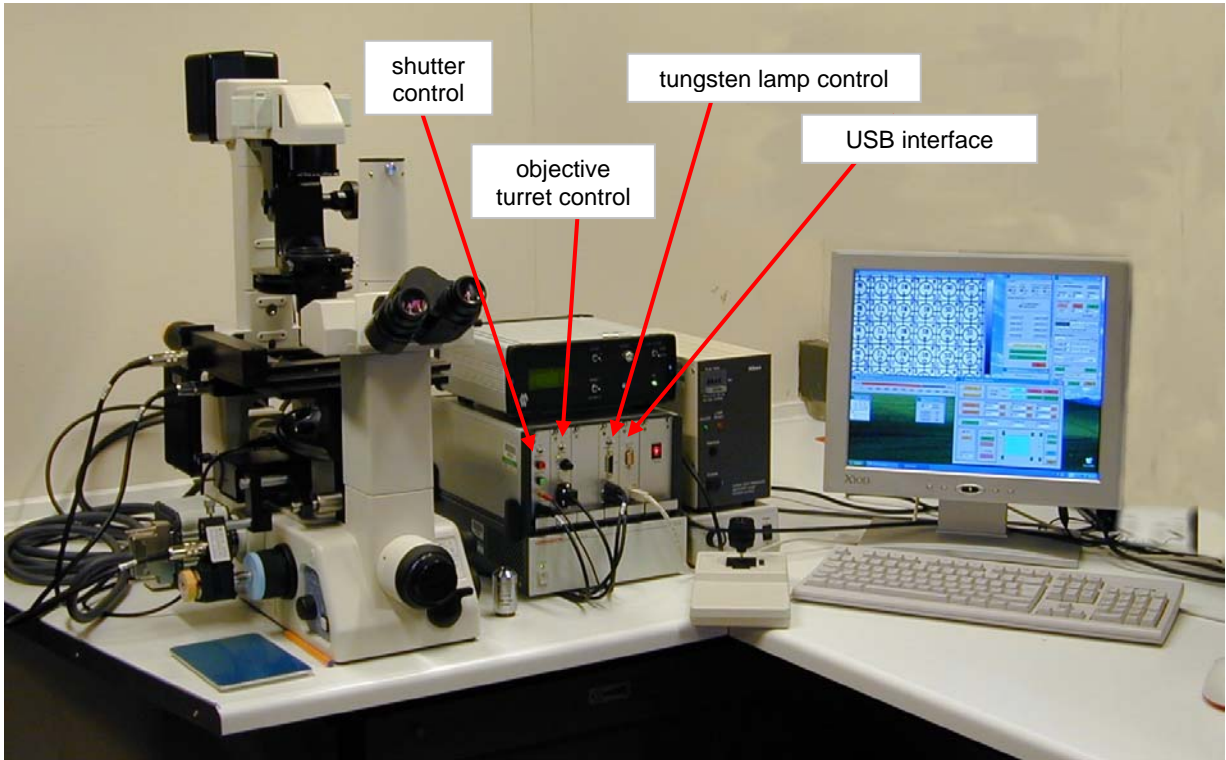
Another example of a TE300/ORCA system, used for off-line imaging of 'microbeam' dishes and incorporating I^2C interfaces to control a motorised objective turret, to control the brightness of the tungsten lamp and to operate the excitation shutter
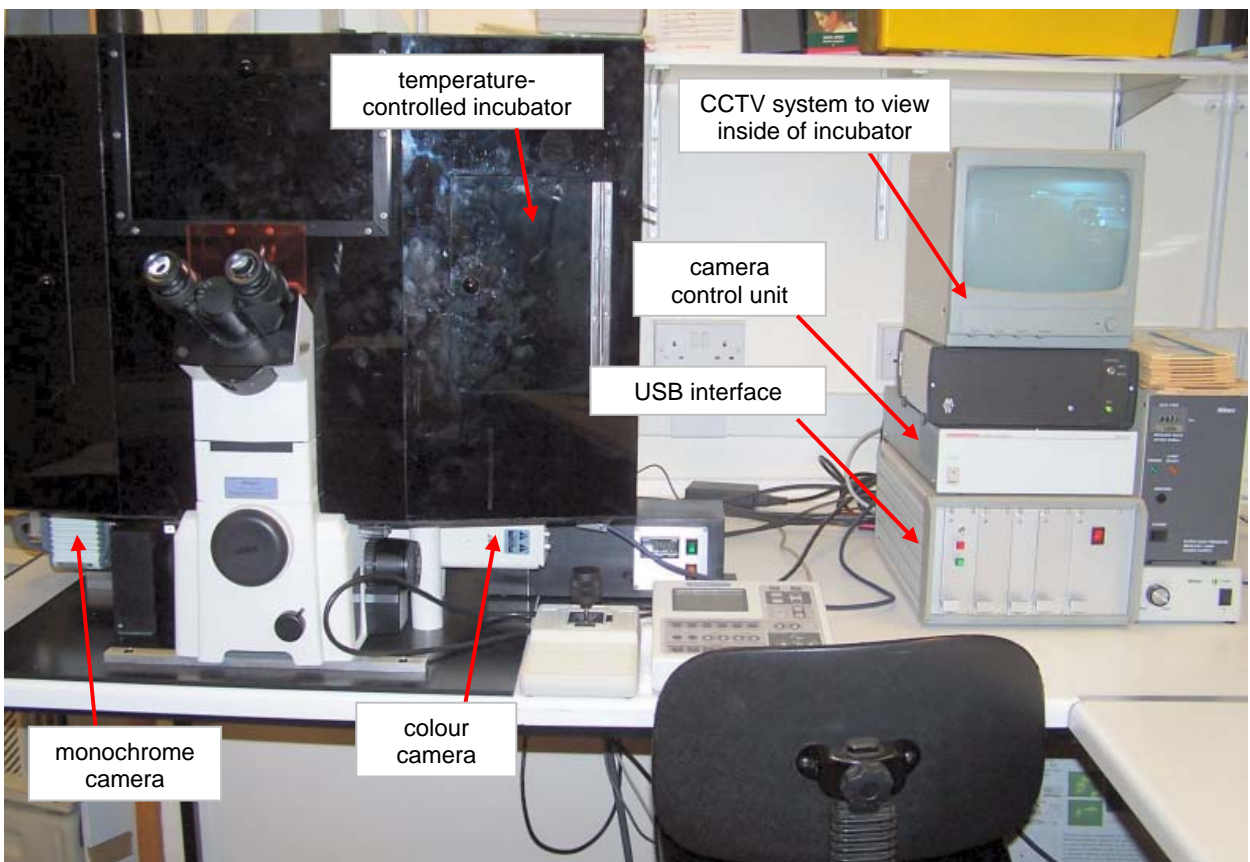
An example of a TE2000 dual camera system (ORCA and KYF 75), used for live-cell imaging. Most of the microscope functions are under software control. The USB/ I^2C interface unit is used to drive a fast shutter, to control power distribution, to control a spectral imaging module and is ready for future expansion.

Examples of 'older' system using analogue cameras; above: system using the cooled Cohu 4900 series ½" camera for fluorescence work; below: system using 3 x ⅓" chip JVC colour camera.

Useful addresses of suppliers

Cameras, imaging:

Mr Brian Reece
BRSL
12 West Mills, Newbury,
Berkshire, RG14-5HG
Tel: 01635 32827
Fax: 01635 34542
http://www.brsl.co.uk

Hamamatsu Photonics UK Ltd
2 Howard Court, 10 Tewin Road
Welwyn Garden City, Herts, AL7 1BW
Tel: 01707 294 888
Fax: 01707 325 777
http://sales.hamamatsu.com

Mr David Tuer
MicroCam Ltd
31 Nearton Road, Swanbourne,
Buckinghamshire, MK 17 0SL
Tel: 01296 720 990
Fax: 01296 720 930

Nikon UK Ltd
Nikon House, 380 Richmond Road
Kingston, Surrey, KT2 5PR
Tel: 0208 541 4440
Fax: 0208 541 4584
http://www.nikon.co.uk

Sensors, motors

Penny and Giles Controls Ltd
Units 35/36, Nine Mile Point Industrial Estate
Cwmfelinfach, Gwent, NP11 7HZ
Tel: 01495 202 000
Fax: 01495 202 006
http://www.pennyandgiles.com

Maxon Motor UK Ltd,
Alberto House, Marino Way, Hogwood Lane,
Finchampstead, Berkshire, RG40 4RF
Tel: 01189 733 337
Fax: 01189 737 472
http://www.maxonmotor.com/

Shutters:

BFI Optilas Ltd
Mill Square Wolverton Mill
Milton Keynes, MK12 5ZY
Tel: 01908 326 326
Fax: 01908 221 110
http://www.bfioptilas.com

Microscope stages

Marzhauser Wetzlar Gmbh u Co. KG
In der Murch 15, Wetzlar
Postfach 2109, Germany D-35579
Tel: 0049 644 191 160
Fax: 0049 644-191 1640
http://www.marzhauser.com

I^{2}C devices

Maxim Integrated Products UK Ltd
Unit 3, Theale Technology Centre, Station Rd
Theale, Berkshire, RG7 4XX
Tel: 01734 303 388
Fax: 01734 305 577
http://www.maxim-ic.com/

USB modules

Future Technology Devices Intl. Limited
St. George's Studios, 93/97 St. George's Road,
Glasgow, G3 6JA,
Tel: 0141 353 2565
Fax: 0141 353 2656
http://www.ftdichip.com

Software

National Instruments UK Ltd.
Measurement House,
Newbury Business Park, London Road,
Newbury, Berkshire, RG14-2PS
Tel: 01635-572-400
Fax: 01635-523-154
http://www.ni.com