# Intravital microscopy

**B. Vojnovic and P. Barber**
**Gray Cancer Institute**

In principle, there is little difference between conventional microscopy methods and intravital microscopy; in practice, however, there are significant differences in the way images are acquired and processed and a number of unusual features are associated with the microscope mechanics and other accessories. This note describes the approaches that we have developed over the last few years at GCI and is intended to emphasise the more 'peculiar' requirements associated with imaging of tissues in live animals; in our case, this is restricted to mice and rats.

Almost all intravital microscopy imaging that we undertake is of tumour tissue and involves tumours grown in 'window' chamber preparations. These allow optical access to the tissue. Further details can be found on the Tumour Microcirculation Group pages. Any data presented here has been acquired from preparations made by Gill Tozer and her colleagues.

The first and most obvious difference between a conventional microscope and one optimised for animal *in vivo* work is the way the 'sample' is handled: animals are thicker than a microscope slide! The stage, the working distance of condensers and objectives are thus likely to be different. The stage in particular benefits from a custom design, where the lower part of the animal is kept either below the objective in the case of an inverted microscope, or where its upper surface is maintained above the objective. This is illustrated in Figure 1. Although we started our work with an inverted microscope system, an upright microscope has proven to be rather more convenient in practice and is generally preferred.

Figure 1: Two of GCI's intravital microscopes. Left: the original system based on an inverted microscope. Right: system based on an upright microscope and featuring a large stage/sample platform.

It is very rare that only microscopy experiments are performed, and most intravital microscopy systems will be used in conjunction with a range of physiological monitoring systems. It is thus advantageous to use a physically large stage to 'hold' a variety of additional sensors, tubing etc. Furthermore, if imaging over extended periods is contemplated, particularly under anaesthesia, some form of thermostatic control of animal core and surface temperature is essential. All these

 

requirements contribute to the need for a customised stage. Although a motorised stage is certainly desirable, manually operated stages are perfectly adequate. However, manual stages should really be fitted with some form of position readout system: it is often desirable to revisit easily and quickly one of several locations in the tissue. This requirement is made somewhat easier by the fact that very high magnifications are not readily possible and hence stage accuracy is of secondary importance.

Unlike the bulk of conventional cell imaging methods, in intravital microscopy, the interest is most often associated with imaging and measurement of dynamic events. This places rather different demands on the choice of cameras, particularly for fluorescence work, and on image – or rather movie – storage requirements. Correspondingly different requirements for control and signal acquisition are thus also dictated.

Although confocal or multiphoton fluorescence excitation microscopy systems offer the possibility of imaging tumour vascular networks in three dimensions, and with a high degree of specificity, more conventional, two-dimensional, optical imaging approaches are useful for more routine work. Moreover, they have the advantage of lower cost and are capable of 'live' imaging and thus useful in the study of highly dynamic phenomena. Three-dimensional imaging methods used at GCI are described in a separate document 'Multiphoton microscopy'.

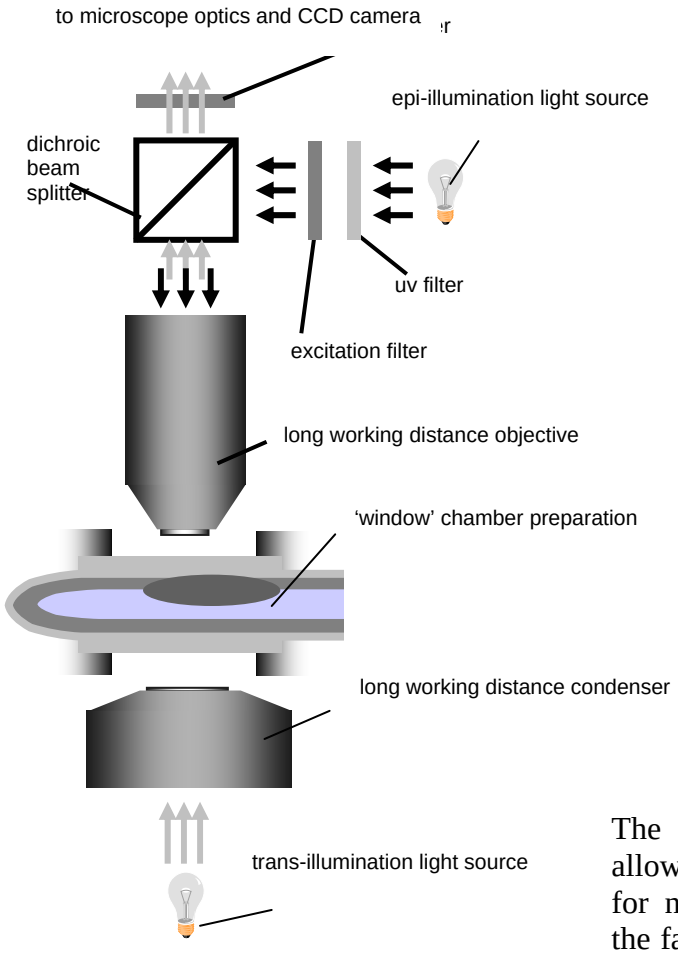


Figure 2: Two-dimensional imaging of 'window' chamber preparations, using conventional microscopy optical components.

For the more conventional techniques, we have found it most useful to utilise just two 'infinite tube length' objectives (4x ??n.a. and 20x ??n.a.) coupled to a Nikon upright microscope, particularly designed for cell physiology work, and to provide a continuously variable magnification (zoom) optical arrangement which views the microscope's image plane and allows post-objective magnification over the range 1-10x. Conventional approaches for trans-illumination are routinely used and readily switched to epi-illumination for fluorescence work (Figure 2). In the latter case, fluorescence intensities are generally low and a more sensitive, monochrome image-intensified camera replaces the more usual colour CCD device.

The availability of epi-illumination also allows the possibility of using this mode for non-fluorescence work by exploiting the fact that light is scattered in tissue. By replacing the usual fluorescence 'cube' with a beam splitter fitted with two crossed polarising filters, vasculature in optically dense tissue, as is often encountered in tumours, can be imaged

 

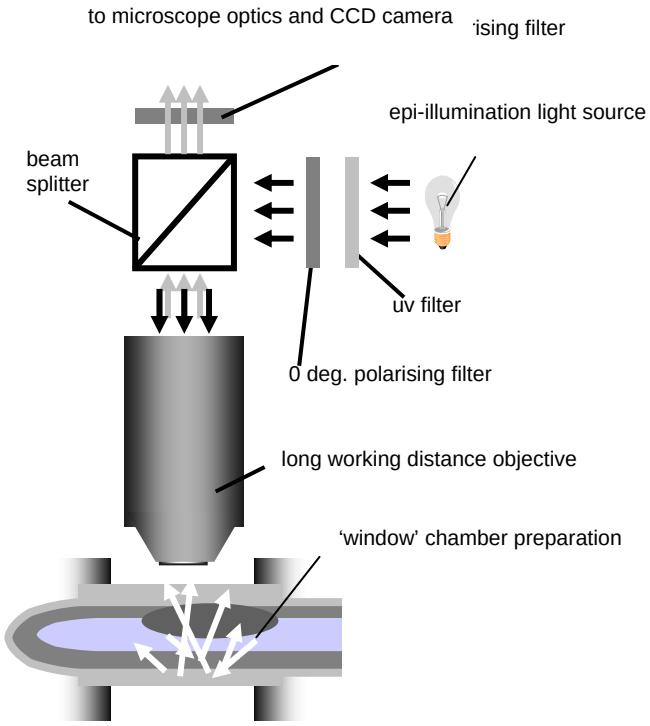


Figure 3: Polarised light epi-illumination optical system, using a 50:50 beam splitter in place of the more usual fluorescence cube. Illumination light is scattered within the tissue and provided trans-illumination of

with improved contrast. This arrangement is shown in Figure 3. The disadvantage is that at least half the light intensity is wasted in the 50:50 beam splitter and that somewhat increased illumination intensities are necessary, since it is the scattered light component that effectively provides trans-illumination light.

Nevertheless, this is a minor problem with modern, sensitive cameras. Exemplar images with conventional trans-illumination and polarised light epi-illumination are shown in Figure 4. In our arrangement, switching between the different imaging modes is accomplished under computer control and we would recommend the use of an electrically operated shutter in the epi-illumination path, synchronised to the image-recording device, particularly when fluorescence excitation is used.

Although high frame rates cameras are currently available, for the majority of dynamic phenomena, video rate (CCIR, 25 frames $sec^{-1}$) systems are quite adequate. Moreover, long-term storage of such images is readily accomplished with digital video recording devices. We have found it convenient to use the 'DV' format, such as provided by a Sony ????? device.

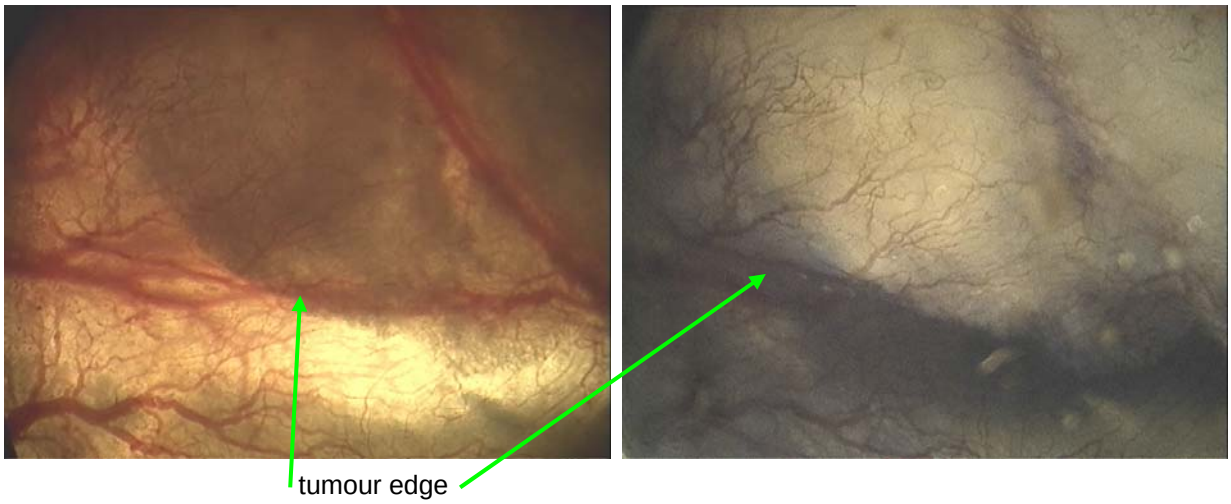


Figure 4: Examples of imaging the same tissue area within a 'window' chamber using transillumination (upper panel) and polarised light illumianation (lower panel). The denser tumour tissue limits available contrast when trans-illumantion is used, while less scattering normal tissue (left and bottom regions of the images) are not so readily observed when polarised light illumination is used.

Although a slight loss of image quality is inherent with this approach, which uses 4:2:2 image compression, this is not a disadvantage in practice, since the ultimate resolution is fundamentally limited by light scattering and out-of-focus light inherent to optical systems which do not use any form of optical sectioning.

While it is certainly possible to perform a range of image processing functions in real time on dedicated hardware and software within a PC, it is often more convenient to perform this on 'simpler' dedicated hardware. We use a rather flexible video processing unit manufactured by BRSL, the XXXX, which combines the functions of a frame grabber, image processor and video overlay unit. This allows easy, mouse-controlled, on-screen measurements (lengths, areas etc.) to be performed, while at the same time providing 'live' image enhancement procedures to be applied, such as contrast stretching and frame averaging etc. A simple video signal matrix allows this processor to be switched in pre- or post-video recording and switching between camera outputs. Still, i.e. 'grabbed', images and measurement data can be transferred to a PC for archiving purposes.

Of course in some instances video framing rates are not adequate for measuring fast events, such as those required to determine blood flow velocities or fluorescent particle flux. For these faster events, we use an optical system that performs fast light intensity measurements from selected regions of the image. This configuration, shown in Figure 5, is placed between the objective and the microscope's tube lens, i.e. in the infinity path.

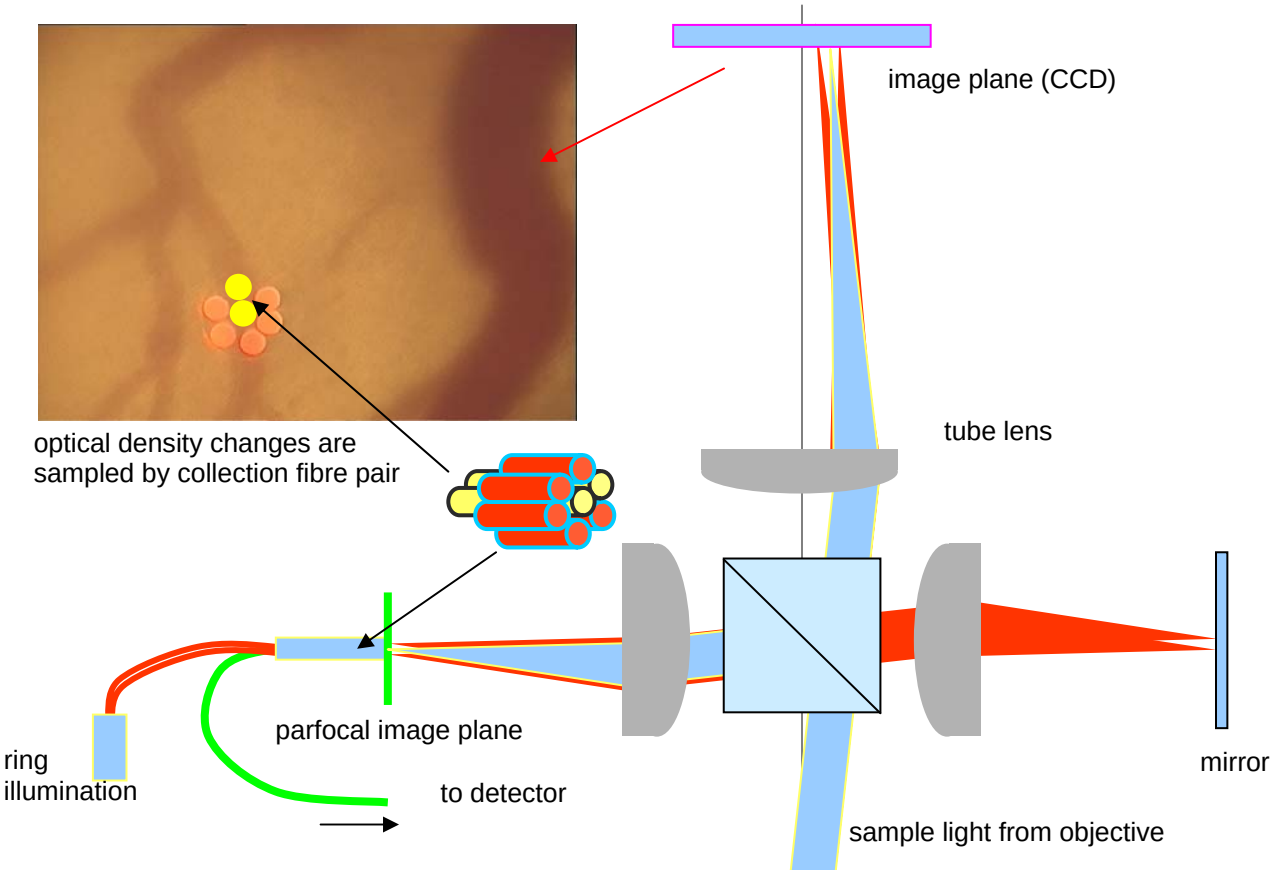


Figure 5: Spot photometry optical system, using a 50:50 beam splitter to produce a second, parfocal image onto o fibre bundle which can be positioned and rotated in the plane (XY, R). Two of the fibres sample the light, while theother five are illuminated. This illumination light passes through an afocal system and returns to the beam splitter so that it is superimposed on the microscope's main image plane. The 'sampled' position can thusbe identified.

The practical implementation of this arrangement is shown in Figure 6. When operating in the transillumination mode, optical density changes, due to the passage of red blood cells, are 'seen' by the optical fibre pair, but the light intensity waveforms are delayed with respect to each other. Since the distance between the fibres is known, their effective separation distance in the objective sample plane is also known. Velocity can thus be determined by dividing this separation by the average delay time between events picked up by the two fibres. This delay time is determined by performing a cross correlation between the fibre signals, as shown in Figure 7.

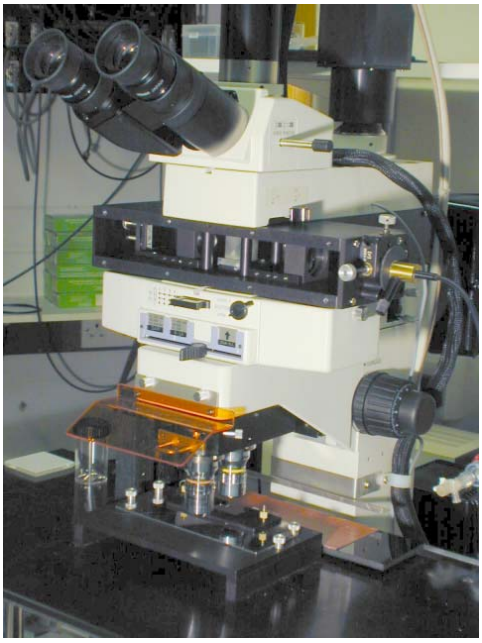

Figure 6: Practical realisation of the optical system depicted in Figure 5, constructed in an assembly placed above the fluorescence cube block and below the eyepieces/camera ports.

The outputs of a pair of photomultiplier tubes are digitised in a 200 kS $sec^{-1}$ A-D converters and correlation analysis is performed in software. A range of other signal processing, smoothing and averaging functions is performed in real time and a 'chart-recorder' type of display of flow velocity is presented.

This type of optical arrangement has the advantage that the portion of the image from which light, i.e. changes in light, are sampled shows up on the microscope output image, by using the 'ring' illumination fibres; light from these passes through the afocal system formed by the mirror and the lens on the right of Figure 5.

The drawback is that the fibre separation is constant, i.e. it can only be changed by inserting a different fibre bundle into the XY-R holder. The range of delay times that can be determined is restricted by signal-to-noise ratio at short times and by correlation sample length at long times. Since the dynamic range of blood flow velocities exceeds 100:1, the range of delay times is comparably large and appropriately practical delay times can only be changed by varying the primary magnification, i.e. the objective.

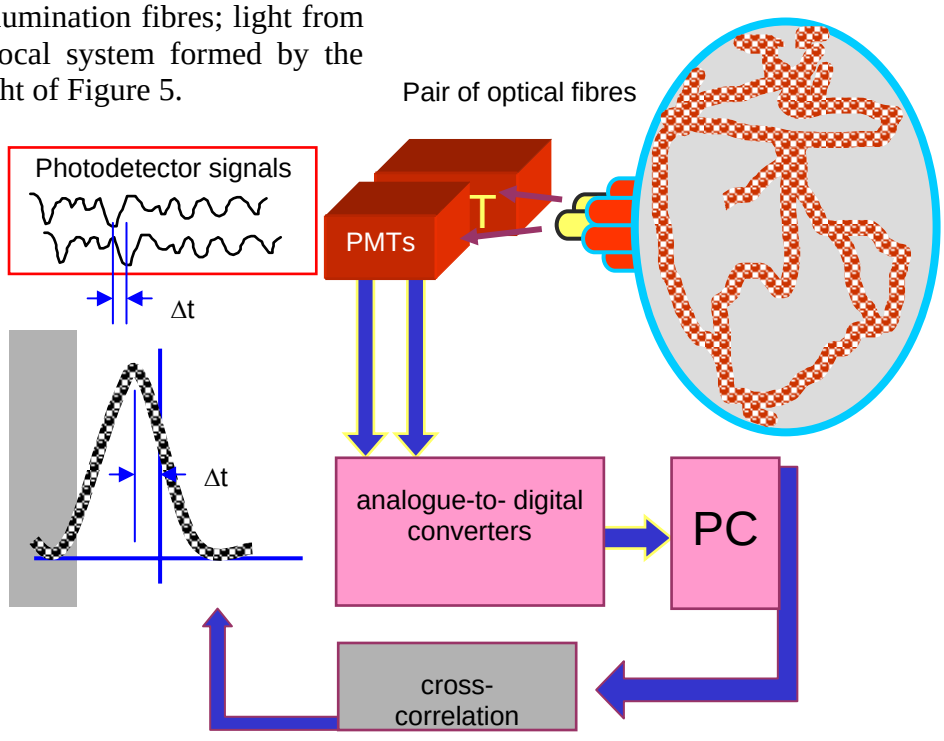


Figure 7: Determination of blood flow velocity by measurement of delay time between optical density changes sensed by fibre pair. Delay time is determined by signal cross-correlation and velocity determined from knowledge of fibre separation in objective focal plane.

Typically, some $10^4$ samples are acquired and results are presented every few seconds to tens of seconds. All these processes are controlled in a dedicated software package developed in-house. In

addition to ‘chart-recording’ of flow, display of other physiological parameters is presented. These include animal temperatures, blood pressure and heart rate. The latter are derived from a pressure transducer/bridge amplifier that has enough bandwidth to record systolic and diastolic pressures. The pressure transducer signal is digitised and the time-varying component is extracted by software high-pass filtering. Spectral analysis on this filtered signal is performed using a Fast Fourier Transform algorithm and the principal component, i.e. the heart rate, is identified and presented on an additional chart recorder display.

Animal temperatures are also recorded and controlled in simple hardware proportional regulators. We use two independent feedback loops. The first controls a heating pad below the animal and using rectal temperature, sensed by a thermocouple as the feedback input. The second loop maintains the appropriate surface temperature, again sensed by a thermocouple, by controlling the power fed to a heating lamp. The lamp is part of a modified ‘angle poise’ fitting and an opaque, but infrared transmissive filter is used to eliminate visible light; these can be seen in Figure 1.

**ME2 Control Software**

Sorry a screen shot is not yet available.

The ME2 control software allows the pc control of the Brian Reece Scientific Ltd (BRSL) ME2 microscopy image processing unit.

The ME2 comprises two older BRSL products, the David and the Advice Colour – hence the confusing interchange of all these names! The David deals with all on screen measurements and overlays and the Advice performs all the real-time image processing including image capture, add, subtract and zoom etc. The computer has a serial interface (usually COM2) with David, and a SCSI connection with the Advice. The single name, ME2, is used here and in the software.

Image Processing
The ME2 has one internal image frame-store.
The PC software has 4 frame-stores (stores 1, 2, 3 and 4).

The contents of the ME2 frame-store can be transferred to the PC by clicking “Grab Image” for the pc store you wish to transfer it to.

“Put Image” transfers the relevant pc store back to the ME2 store.

The contents of a pc store can be viewed at full size with the “Display” button.

The “File” pull-down menu for each pc store allows you to save the image to disk, load another image from disk or print an image. Each menu only works on the store it is next to.

Once an image has been put-back into the ME2 store the image processing functions can be used. The pull down menu allows you to:

| | |
|---|---|
| View Image | - view the ME2 store. |
| Split | - split screen between ME2 store and live. |
| Flash (Nictitate) | - flask between ME2 store and live. |
| Add | - Add ME2 store and live images. |
| Subtract | - Subtract ME2 store from live image (or vice versa). |

 

The “Subtract” function will also work without an image in the ME2 store. In this case, live subtraction is performed between subsequent frames in real-time. Click “Live View” and then select “Subtract”.
Other functions that work on the live image are: (N.B. the ME2 store is overwritten)

| | |
|---|---|
| Zoom | - Zoom into the live image |
| Noise Reduction | - Reduce noise in live image |
| Negative Image | - change black for white etc. |

The “Live View” button returns to normal live view with no processing.

The “ME2 Controls” button gives full control of all ME2 functions including flash rate, split position, zoom position etc., plus control of image brightness and contrast.

Measurements
An on-screen measurement taken with the ME2 can be transferred to the pc for use in a calculation or storage in a spreadsheet. This is done by pressing the middle button of the ME2 mouse. Currently only the Length measure and objective in use are transferred and appear on the pc screen. (N.B. for the MicroVas program the length and objective are used in the blood flow and speed calculations).