

An Optical Projection Tomography Scanning Microscope

Alasdair Edgar

Anatomy is often presented in two dimensions, as photographs, but by presenting anatomical information in three dimensions we can more rapidly visualise how a complex structure is arranged. 3D is especially beneficial when examining branching structures such as nerves and blood vessels that in a 2D picture may appear to join when in fact they cross.



Optical Projection Tomography (OPT) is an imaging technology for biological specimens that generates 3D images from multiple two dimensional images taken from different directions and was first reported by James Sharpe's research group in the magazine Science in 2002. For many purposes OPT microscopy can replace the laborious task of reconstructing 3D structures from serial histological sections. We are using a Bioptonics 3001 microscope to study fixed animal specimens and tissues that are up to 1 cm in diameter, but it does not provide a sub-cellular resolution (Figure 1).

This OPT microscope is the first commercial production model and is currently used only for research purposes; other OPT microscopes are usually custom built and modified by researchers with a view to developing and improving the microscopy and its utility.

The histological specimen is cleared to make it transparent in most areas and suspended in an

OPT microscopy can replace the laborious task of reconstructing 3D structures

optically suitable medium in a quartz cuvette in which to rotate the specimen. The OPT microscope records both absorption and emission profiles. Brightfield OPT microscopy records the morphology of the remaining darker areas of the specimen using transmitted white light as shadow images. The OPT microscope is also able to record autofluorescence emitted from the specimen back along the optical path using a UV-lamp as a light source. The degree of autofluorescence in a specimen is affected greatly by the fixation protocol of the tissue since aldehyde fixatives, such as glutaraldehyde, react with proteins and amines creating fluorescent products.

Prior to examining the specimen in the microscope, patterns of messenger RNA expression and protein

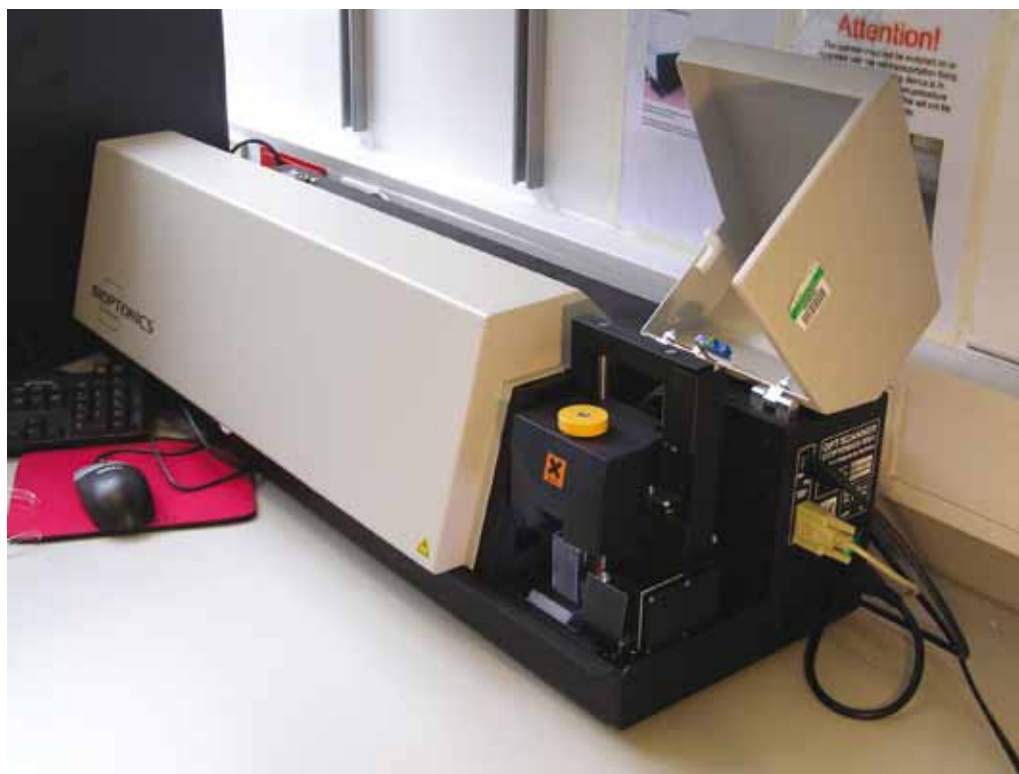


Fig. 1. The Bioptonics 3001 scanning microscope.



Fig. 2. An agarose block containing a specimen.

distribution are developed. Whole-mount *in situ* hybridisation is used to define mRNA (or gene) expression and immunohistochemistry using antibodies is used to define the localisation of specific proteins. Both techniques can be shown by the development of coloured stains or the use of fluorophores. Importantly, specimens labelled with coloured stains, such as the brown diaminobenzidine (DAB), are seen as a shade of grey when seen as shadow images through the microscope. Another common method used is the detection of alkaline phosphatase activity using 5-bromo-4-chloro-3-indolyl-phosphate and nitro blue tetrazolium which produces a purple precipitate within the tissue (the BCIP/NBT method).

The OPT microscope is a marriage of a standard fluorescence microscope laid on its side coupled with the specimen rotation component of a micro computerised tomography (CT) scanner. Overall, it is an unpretentious grey box, and oddly for a microscope, it does not have eyepieces to view

the specimen, relying on a computer screen to display the images. If you are thinking about getting one there are a few things to consider. Although a scanning instrument, it does not need to be situated on anything other than an ordinary laboratory bench. However, care has to be taken to avoid having other instruments that cause vibrations, such as centrifuges, on the same bench and not to slam drawers and doors. Similarly, if the OPT microscope has to be moved, then to avoid damaging the instrument the electronic rotation component scanning head must be securely locked in place.

Relative to other graphic applications the computing system required is not onerous, being PC-based, but it does require a good quality graphics card, and plenty of file storage space. A one channel scan of a specimen takes up about 0.6 GB, the subsequent reconstruction another 0.6 GB and a derived movie up to 0.3 GB, depending on your choice of resolution. A high resolution scan has twice the resolution of a standard scan, but because it is in 3D requires eight times as much storage space. The

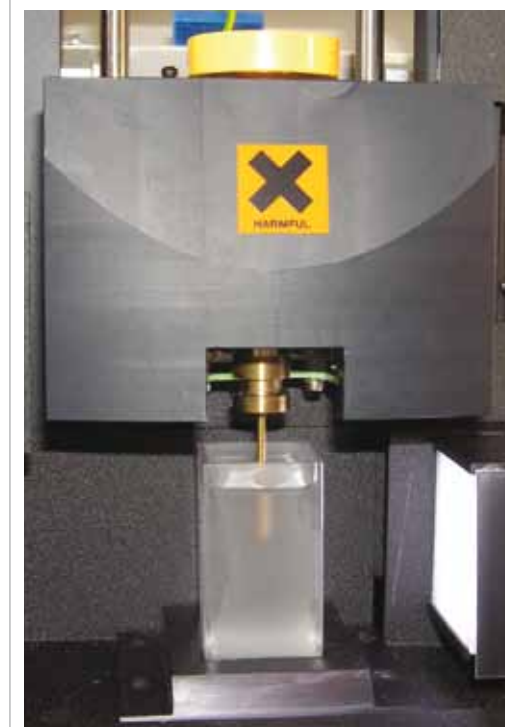


Fig. 3. Calibrating the microscope with the alignment pin.

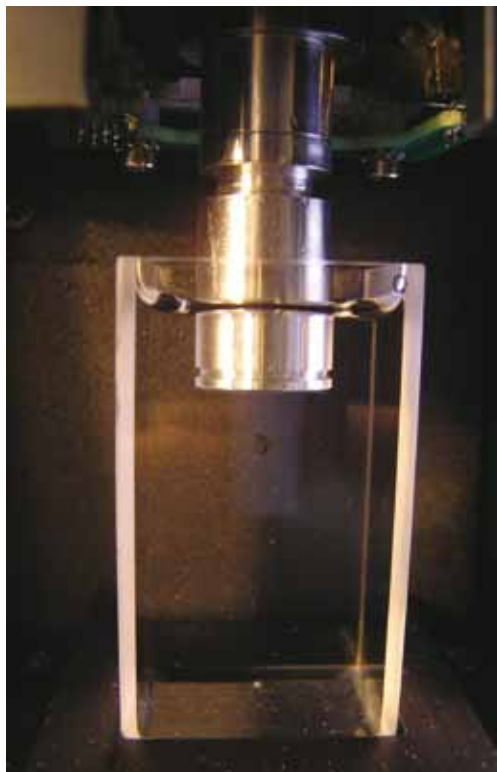


Fig. 4. The inverted specimen suspended in the clearing agent. Note: the cuvette is turned 90° for the purpose of illustration.

original instrument came with 0.5TB of storage, and we now use 1TB external drives for storage and back-up purposes.

The preparation of specimen such as an adult mouse kidney involves whole-mount immunohistochemistry and/or *in situ* hybridisation as required. After post-fixing in 4% paraformaldehyde solution the specimen is washed into phosphate buffered saline (PBS) and mounted in a supporting block of 1% low-melting point agarose. The tricky bit is sticking the agarose block on a magnetic mount with a cyanoacrylate adhesive (superglue). The block is trimmed with a sharp blade to give a smooth surface with many facets, leaving a few millimetres of agarose around the specimen (Figure 2).

Over the course of a few days, the specimen in its agarose block is dehydrated in 100% methanol, turning it opaque, and is subsequently placed in a histological 'clearing agent' (a liquid) to make the specimen transparent which optically matches the refractive index of cell membranes. Normally, this



Fig. 5. Film strips of a frog tadpole (8.5 mm long): transmitted white light images with the corresponding emitted autofluorescence images through the GFP1 filter set shown below. The frames shown are 13.5° apart.



Fig. 6. Horizontal slices, 270 μ m apart, from an unstained adult mouse brain reconstructed from the transmitted white light channel are shown in a false-colour intensity scale.

is a mixture of 2 parts of benzyl benzoate to 1 part benzyl alcohol (BABB), which has a refractive index 1.51.

As BABB contains harmful chemicals, it must be dispensed in a fumehood. There will be some exposure to the vapour during specimen transfer to the microscope and from the surface of the liquid in the cuvette. However, since the vapour pressure of BABB is very low this should not cause any harm as long as the air in the laboratory is replenished. BABB is a solvent and effectively dissolves plastics such as computer keyboards. Any spilt BABB must be cleaned up with absorbent tissues soaked in methanol.

Before scanning a specimen, the scanning microscope needs to go through some alignment and calibration checks using the alignment pin (Figure 3).

The specimen stuck to the mount is inverted and attached by magnetism to the rotating plate above the cuvette in the OPT microscope. The door to the specimen chamber is closed and the lowering of the specimen into the cuvette is controlled using the Skyscan scanner software on the computer (Figure 4).

Things do go wrong: BABB or methanol trapped between mount and rotating plate affects the rotation of the specimen; the superglued surfaces may come apart, but after trimming can be re-stuck; air bubbles trapped within the specimen show up all too well on the scan.

After centring the specimen, the magnification and exposures are adjusted. Focusing is tricky. The lens has a limited depth of focus, which because of the thickness of the specimen, means that some of the specimen is out of focus. We focus on a sharply defined structure that is about half way between the front of the specimen and its central axis (usually fluorescence from a blood vessel serves well), so as the specimen is rotated all of it is in focus for at least part of the 360 degree scan. The image is recorded by a CCD-Camera with a maximum resolution 1024 x 1024 pixels. A uniform illumination is provided by a panel of white-light emitting diodes behind a frosted screen and, at longer exposures, the white light can be supplemented by infra-red light that penetrates deeper into the tissues of dark specimens.

During scanning, the specimen is rotated 360 degrees around its perpendicular axis in steps of less than one degree, usually 0.9 degree. After each step, a delay allows for any wobbling of the agarose to cease and a still picture is recorded. As these images are shadows they are viewed on a monochrome intensity scale and falsely coloured later. Similarly, fluorescent images of the specimen are taken. The light source for fluorescence is a long-life ultra-violet metal-halide bulb that is still burning strong after a few years of microscope use. The microscope is fitted with three filter sets that cannot be easily changed; a Green Fluorescent Protein (GFP) 1 filter with an exciter 425nm/40 nm, emitter LP475 nm, a GFP+ with an exciter 480nm/20 nm, emitter LP515 nm and a Cy3 with an exciter 545nm/30 nm, emitter 610/75 nm. Usually, four hundred pictures are taken

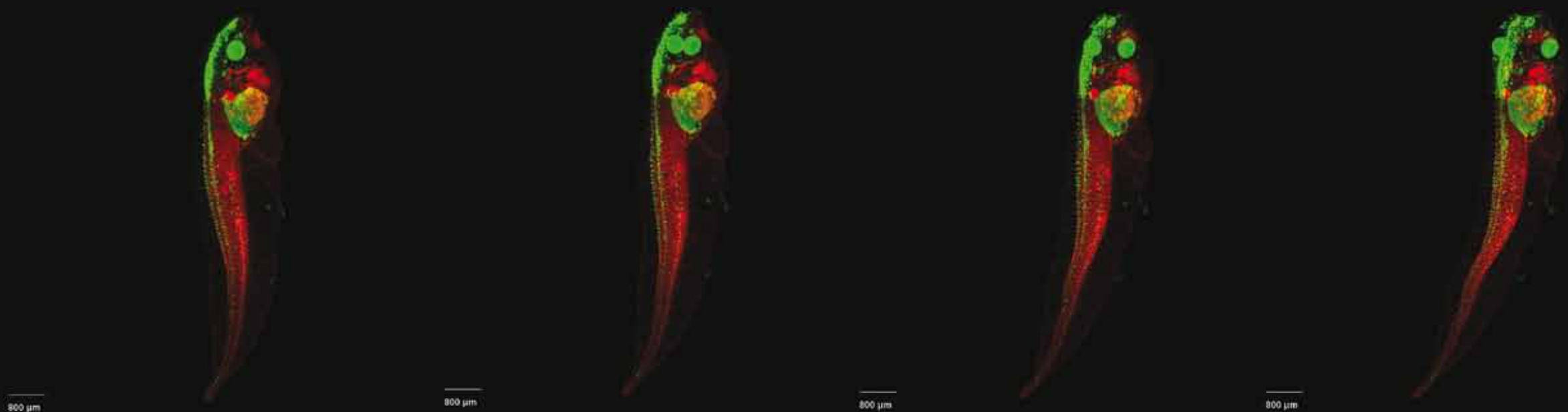


Fig. 7. A maximum intensity projection of the frog tadpole is illustrated with the transmitted white light channel in green and the GFP channel in red.

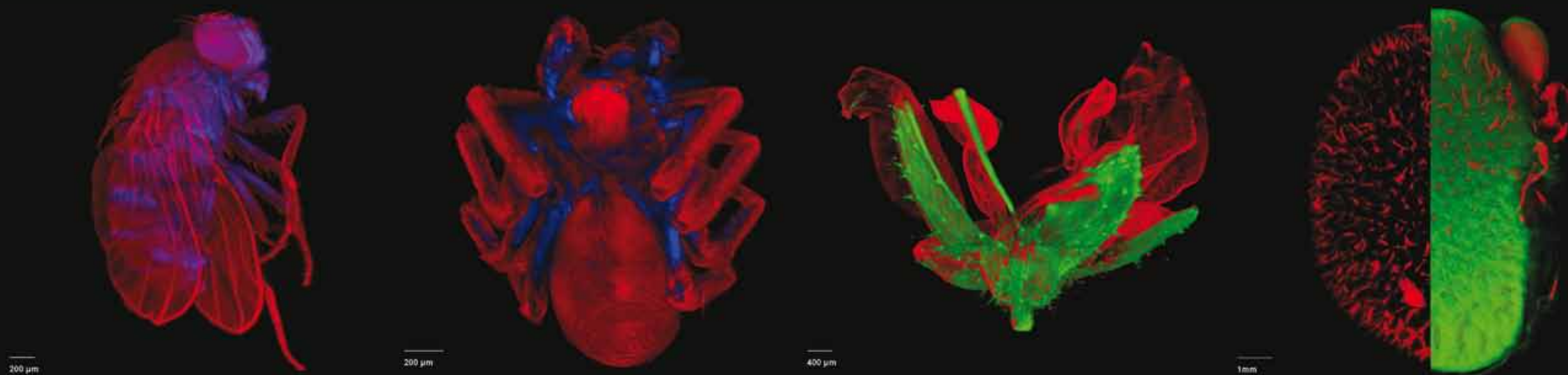


Fig. 8. Images of unstained specimens, left to right: a fly, maximum intensity projection with white light shown as red and autofluorescence on the GFP channel as blue; a spider, rendered image with white light shown as red and autofluorescence on the GFP channel as blue; a flower with white light shown as red and autofluorescence on the GFP channel as green and a kidney from an adult mouse with white light shown as red and autofluorescence on the GFP channel as green (half the picture).

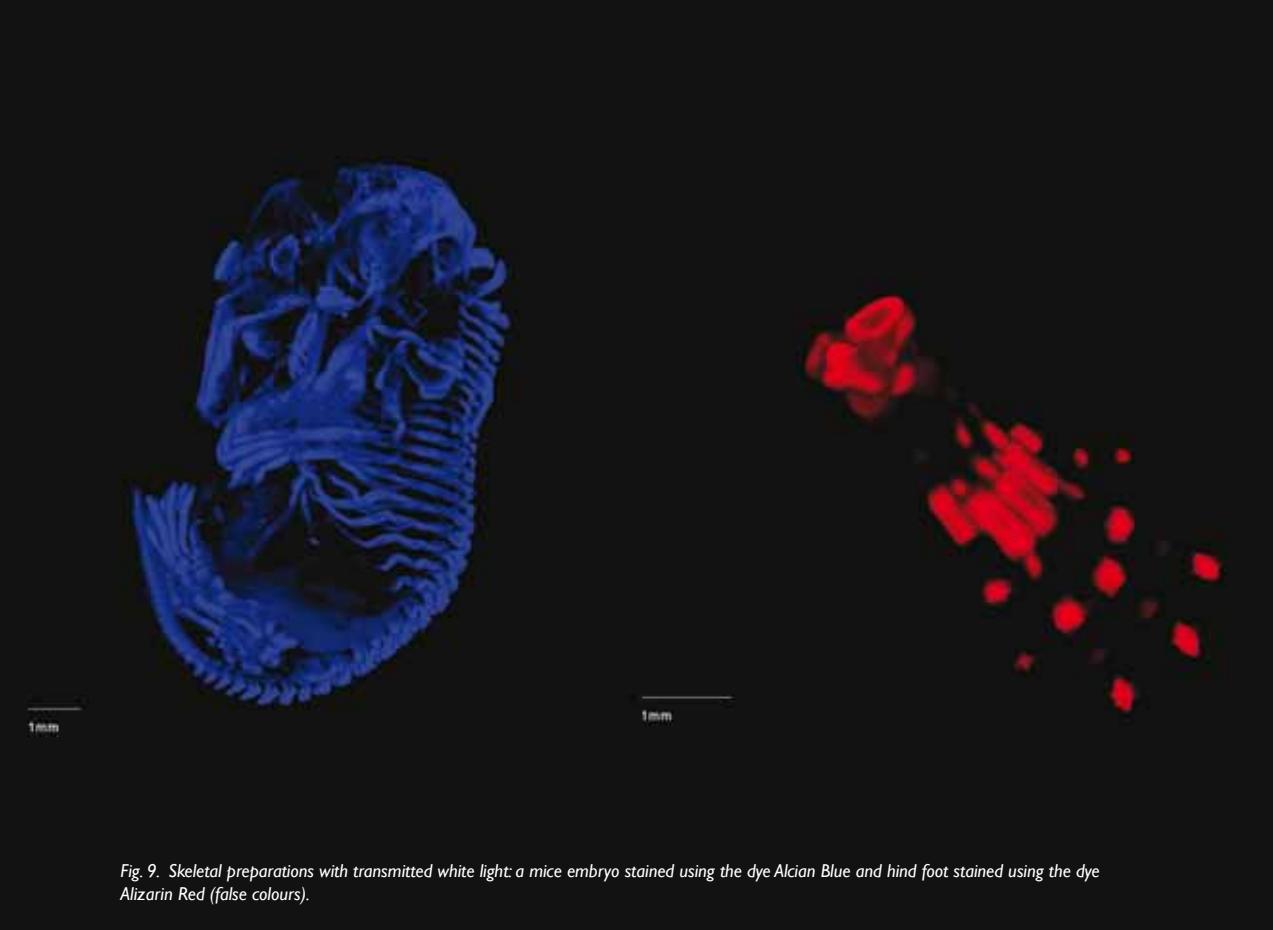


Fig. 9. Skeletal preparations with transmitted white light: a mice embryo stained using the dye Alcian Blue and hind foot stained using the dye Alizarin Red (false colours).

per scan with transmitted light exposures being 5-20 ms and fluorescence exposures being much longer at 100-500 ms. On exposure to UV light, a certain amount of bleaching inevitably occurs between the first and the last frame exposure. These scans are done sequentially, allowing sufficient time for a tea break. The example shown in the film strip is of a frog tadpole (8.5 mm long) where the dark pigment in the eyes and the melanocytes are visible in the transmitted white light channel (Figure 5 and Movie 1).

In the filmstrip the bright autofluorescence of the internal organs is seen through the GFP filter set; the frames illustrated are 15 frames apart (13.5°) (Movie 2).

These 400 2D photographs per channel are saved as TIFF files and the specimen removed. At this stage we can either scan more specimens or proceed to the reconstruction process which does not require the microscope.

The raw data in an image gives you the 2D location and the intensity of the light signal coming through the specimen, but it does not tell you precisely from where inside the 3D specimen that information came. These 400 2D images are processed by computationally reconstructing horizontally slices through the stack of image files to generate 3D voxel information using the reconstruction 'NRecon' software provided. A voxel is like a pixel, but for a volume rather than a 2D surface, i.e. a volume element. This reconstruction takes all the information (the so called 'scattering data') in the 2D images taken round the object, and a mathematical back projection transform is used to 'predict' a reconstruction of the original density of the object from the scattering data.

We generate a few test reconstructions for a single horizontal slice of the specimen using different correction factors and determine, by eye, the most suitable one to correct for misalignment and that correction factor is used to reconstruct the whole

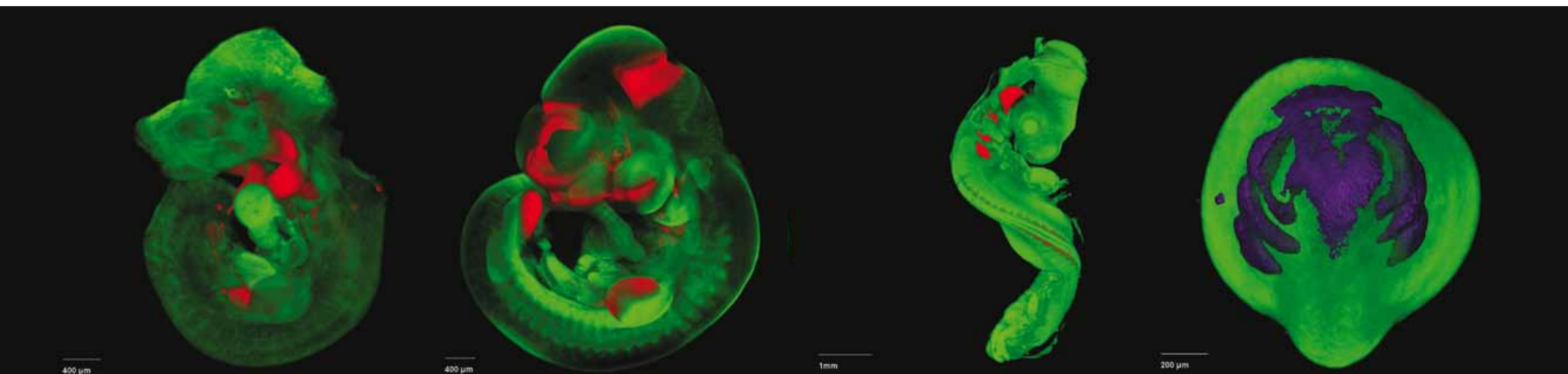


Fig. 10. Gene expression patterns using whole-mount in situ hybridization and developed to show a visible signal. The anatomy is autofluorescence shown as green and the signal is transmitted white light shown as red. From left to right; two mouse, a chick and a frog embryos show contrasting patterns of genes expression; in the frog embryo the signal is shown as iso-surface as a dark purple colour.

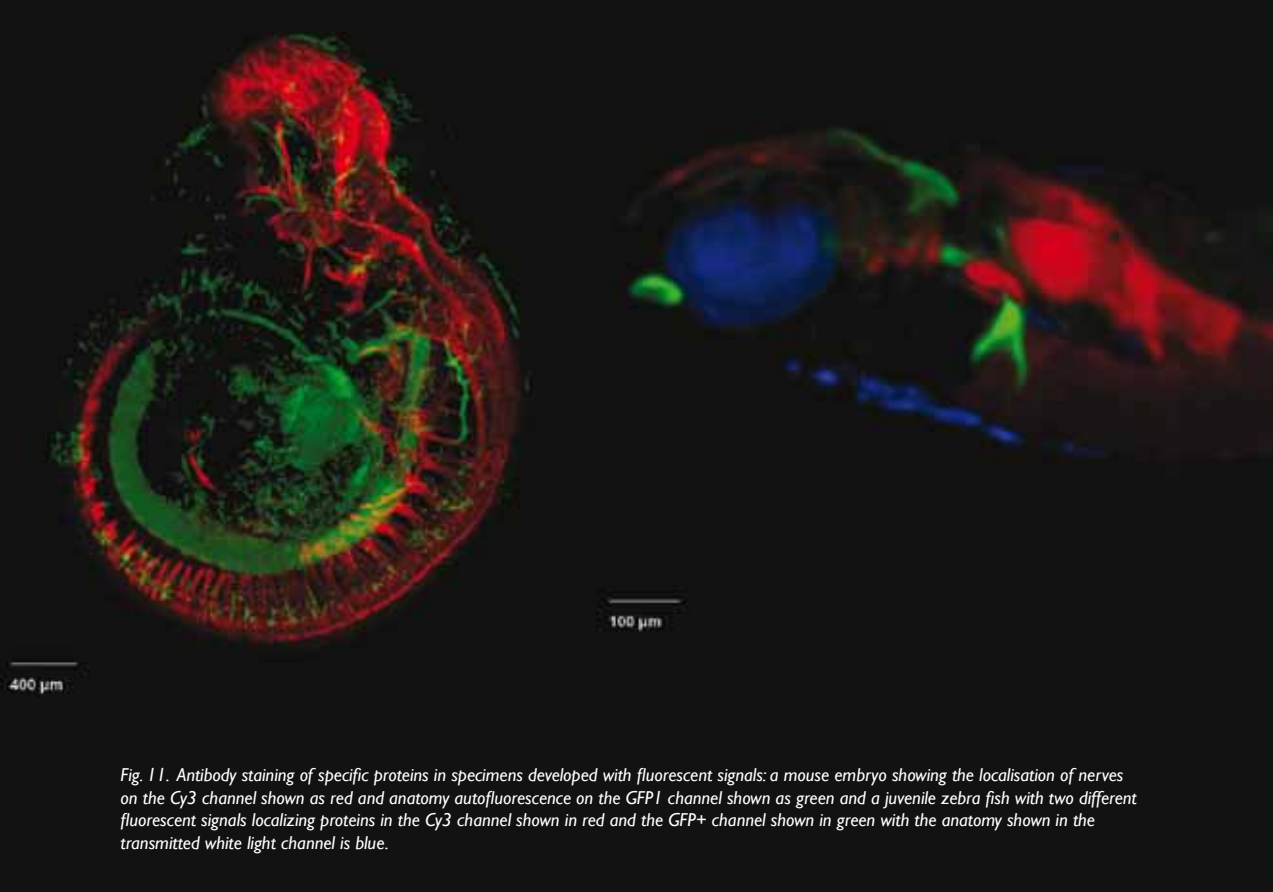


Fig. 11. Antibody staining of specific proteins in specimens developed with fluorescent signals: a mouse embryo showing the localisation of nerves on the Cy3 channel shown as red and anatomy autofluorescence on the GFP1 channel shown as green and a juvenile zebra fish with two different fluorescent signals localizing proteins in the Cy3 channel shown in red and the GFP+ channel shown in green with the anatomy shown in the transmitted white light channel is blue.

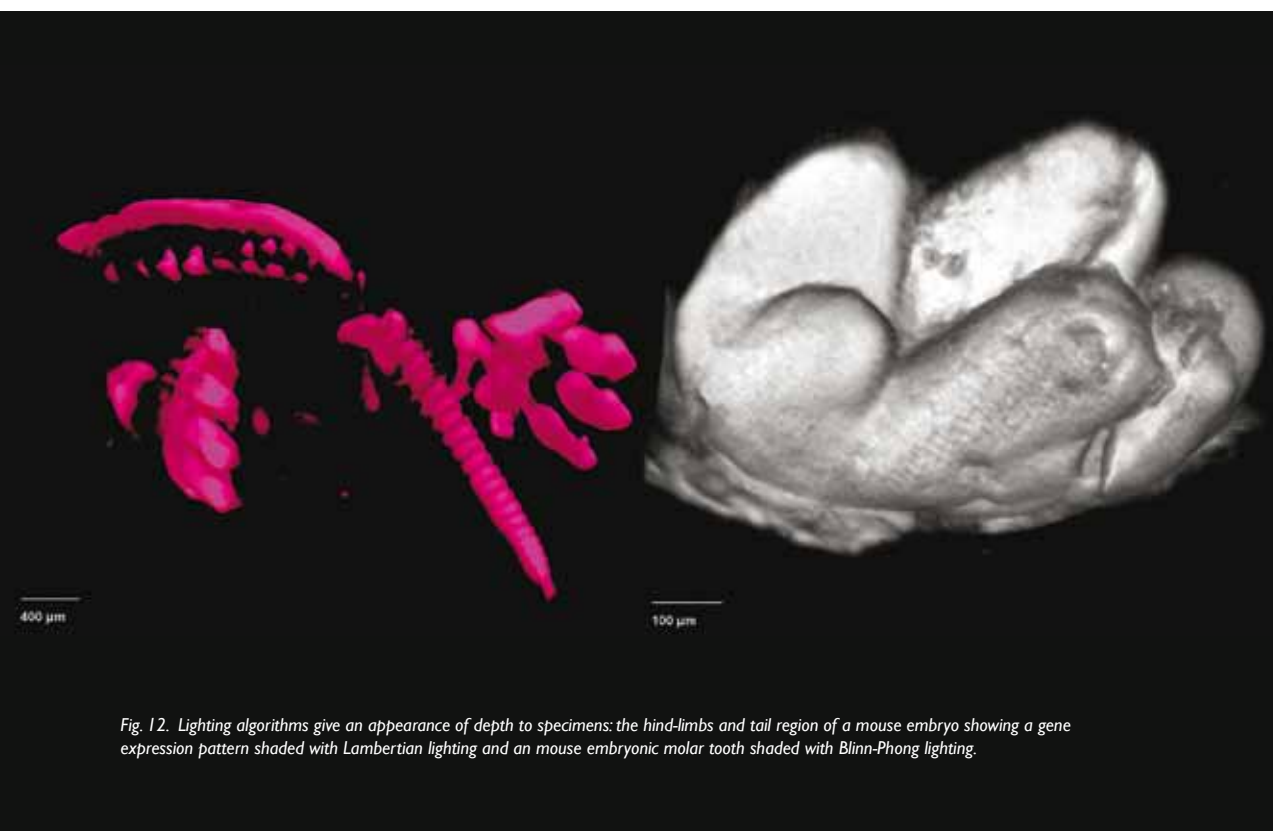


Fig. 12. Lighting algorithms give an appearance of depth to specimens: the hind-limbs and tail region of a mouse embryo showing a gene expression pattern shaded with Lambertian lighting and an mouse embryonic molar tooth shaded with Blinn-Phong lighting.

specimen. Performing a reconstruction takes about 40 minutes per channel, so reconstructions are usually run overnight in a batch. Examples of the reconstructed horizontal (transaxial) slices (13.5 μ m thick and 270 μ m apart) through an unstained adult mouse brain that was illuminated with transmitted white light, are shown in a false-colour intensity scale (Figure 6 and Movie 3).

The next stage is to manipulate the virtual specimen in 3D and zoom in and out using the 'Bioptonics Viewer' software. A single grey-scale will suffice if you are only looking at the anatomy of the specimen. Usually, we integrate data from two or three channels in the rendering window. These are loaded using false colours, red, green and blue, to distinguish the channels; which is not ideal for the colour-blind. Usually we use one channel to show the outline of the specimen and designate this the 'anatomy' channel, and another to show the location, or pattern, of expression of a particular gene and this is designated the 'signal' channel. After setting the magnification and starting orientation you can begin exporting movie frames. The combination of the two channels from the frog tadpole is illustrated (Figure 7 and Movie 4). The transmitted white light is shown in green and autofluorescence from the GFP1 channel is shown in red. This is a maximum intensity projection i.e. the brightest pixel in a ray passing through the specimen is displayed; no rendering of the image has been carried out.

In the rendering window, you balance the combined images to show the salient points of your discoveries. It is important to stress that during your manipulations of the images during the rendering process, you are not adding information to the image, but rather, taking away a background signal and adjusting the contrast and brightness of the opacity and luminosity of the image; basically producing an aesthetically pleasing image. The opacity is a measure of how light passes through your sample; higher opacity of signal channel will make that pattern appear more solid and lower

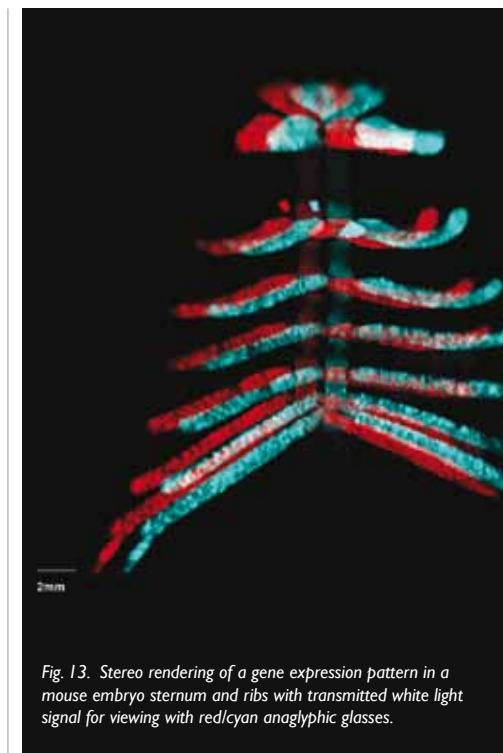


Fig. 13. Stereo rendering of a gene expression pattern in a mouse embryo sternum and ribs with transmitted white light signal for viewing with red/cyan anaglyphic glasses.

opacity of anatomy channel will make the outline of the specimen appear more transparent, thereby ensuring the visibility of the signal channel. The luminance is essentially how bright the sample appears to the eye; using a dark background colour usually requires an increase in luminance of both channels to obtain a satisfactory image. For unstained specimens using just a combination of transmitted white light and autofluorescence shows details that were not previously visible (Figure 8).

Histological specimens can be stained for cartilage using the dye Alcian Blue and for bone using the dye Alizarin Red (Figure 9).

The main usage of OPT has been the mapping of gene expression using whole-mount in situ hybridization during embryo development and for examining differences between normal, or wild type and mutant embryos (Figure 10). The frog embryo shows an iso-surface, which is a calculated surface, where voxels with an intensity above a given threshold are joined together.

Whole mount immunohistochemistry is used to stain specific proteins in an embryo using antibodies (Figure 11 and Movie 5).

Lighting algorithms and lighting directions can be applied to give an appearance of depth to specimens by adding shading, as illustrated by the 'Lambertian' lighting of the hind-limbs and tail region of a mouse embryo showing a pattern of gene expression and the 'Blinn-Phong' lighting of an embryonic molar tooth dissected out of a mouse jaw (Figure 12).

The 3D experience can be enhanced with the use of stereo rendering and cheap coloured anaglyphic 'glasses' (Figure 13 and Movie 6), as supplied with issue 11 of infocus.

After completing the rendering process, the output is a series of computed still frames showing the specimen rotated about a particular axis of your choice. Here, various techniques can be employed to emphasis your scientific finding: rotation about all three axis; symmetrical specimens can be cut in two, showing the anatomy and signal on one side, leaving just signal on the other side; moving slices

can be made through specific channels and channels can be faded out and in. Finally, these still frames are concatenated into movie files using software such as 'QuickTime Pro' or 'Windows Movie Maker'.

As shown here, still frames from the OPT movies can be displayed in the text of an article and movies in a supplement in the electronic version of the article. These movies are usually of a reduced quality, being compressed, so that they are suitable for speedy downloading and sending as an e-mail attachment. The use of OPT movies in talks and presentations often requires the use of compressed movies since they load and play much faster. However, the quality is usually sufficient to display the relevant structures.

Acknowledgments

I thank all the researchers who have provided me with the specimens: Jackie Tabler, Tian Yu, Marc Dionne, Chloe Edgar, Chris Healy, Wei-yuan Yu, Jenny Gardiner, Donald Bell, Triona Bolger, Subreena Simrick, Robert Knight, Pip Francis-West and Heather Rogers.

The OPT movies featured in this article can be viewed online at www.rms.org.uk.

Movie Captions

Movie 1: A frog tadpole transmitted white light shadow images.

Movie 2: A frog tadpole autofluorescence images through the GFP1 filter set.

Movie 3: Reconstructed Horizontal slices from an unstained adult mouse brain from the transmitted white light channel.

Movie 4: Frog tadpole: maximum intensity projection.

Movie 5: Zebra fish: two emitted channels, red and green; transmitted, blue.

Movie 6: Stereo rendering of a mouse embryo sternum (red/cyan anaglyphic glasses).

Alasdair Edgar

King's College London, UK
alasdair.edgar@kcl.ac.uk



Dr Alasdair Edgar received his doctorate in Biochemistry from King's College London in 1989. He is presently Senior Scientific Officer in the Department of Craniofacial Development in the King's College London Dental Institute at Guy's Hospital and is a member of the Anatomical Society of Great Britain and Ireland.