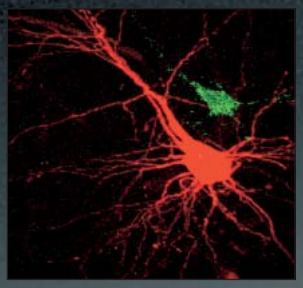
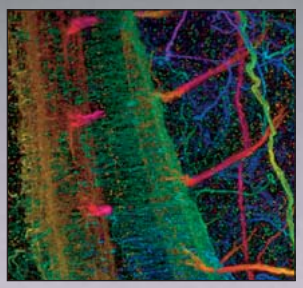
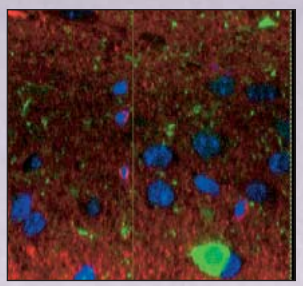
Apr.
2004
No.17

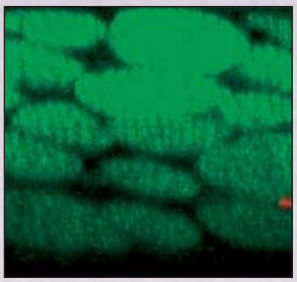
CONFOCAL APPLICATION LETTER



reSOLUTION







GLYCEROL OBJECTIVE

Leica
MICROSYSTEMS

Glycerol Objective

Leica HCX PL APO 63x / 1.3 GLYC CORR CS (21° C)

Sample differences

When considering the refractive indices of biological samples, fluorescence microscopy techniques present us with a wide variety of challenges. In conventional and confocal microscopy, we can identify three broad classes of specimen: animals, plants and bacterial. Each of these has a wide variety of preparation methods, which include, whole mount, slice and culture. We can broadly subdivide these into three main preparation types:

Specifications:

- 63x magnification
- 1,3 numerical aperture
- 280 µm free working distance
- 80% glycerol / 20% water mix immersion
- Correction collar for 21° C standard temperature
- UV-correction optics available (Ar-UV, 405 nm)

Messages

- Best resolution by reflective index match of glycerol / water (80 / 20%)
- No aberrations
- High NA
- Extra long free working distance
- Ideal for thick specimens: drosophila, muscle etc.

1. Living specimen: These are e.g. cultured cells in Petri-dishes (a few microns thick and quite transparent), cultured tissue (up to 30 microns, but still transparent), brain slices (several hundreds of micron thick, with medium transparency) and animals or plants (very thick and often including pigments or non-transparent materials). To maintain viability, most living preparations are mounted in aqueous solutions and buffers. Buffers solutions, in general, have a refractive index close to water (1,33).
2. New methods of fixation: cultured cells, tissue slices of thick specimen as well as whole mount embryos (see Fig. 1) mounted in Moviol, Vectashield or similar mixtures containing water and glycerol plus various chemicals, e.g. antifade and preservation substances. These media have refractive indices close to that of glycerol (1,47). A more detailed description is given below.
3. Classically fixed specimens: thick tissues, and pieces of plants, embedded in resin, Canada balm or glycerine-gelatine. Here the mounting media show a refractive index which matches that of glass. As immersion medium selected oils are used, which again match the glass index at 1,51.

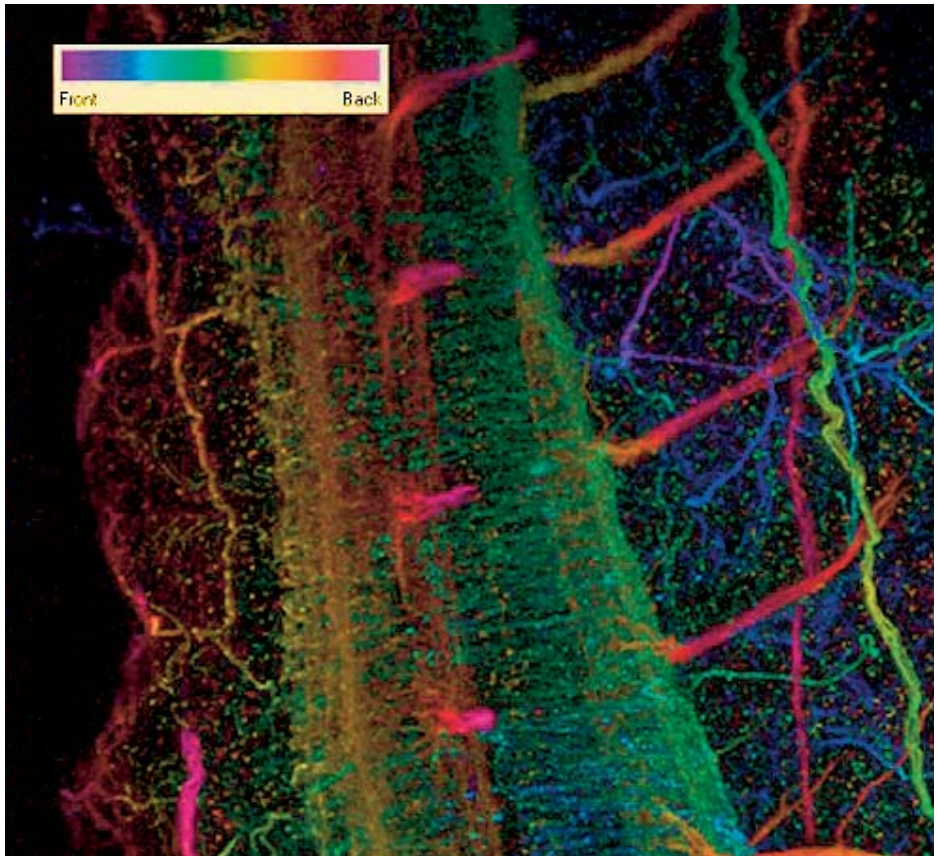


Fig. 1: Nerve system of *Medaka* embedded in glycerol/water (80/20%). Immunofluorescence label of acetylated tubulin with Alexa 594. Recorded stack: 170 µm.
Maximum projection displayed by depth colour coding.
Courtesy of Dr. Martina Rembold, Group Jochen Wittbrodt, EMBL, Heidelberg

Refractive index – basics

The refractive index is a value calculated from the ratio of the speed of light in a vacuum to that in a second medium of greater density. Its difference to the speed of propagation of light rays in a vacuum (air) is given by a factor that is called the refractive index **n**.

As you can see from Fig 2, changes in refractive index cause a diffraction of the light beam. This will occur at all locations, where the index changes. In practise this will occur not only at glass-medium interfaces, but also at membrane-plasma interfaces or lipid droplets, starch grains and so on in cells and tissues (Fig. 3).

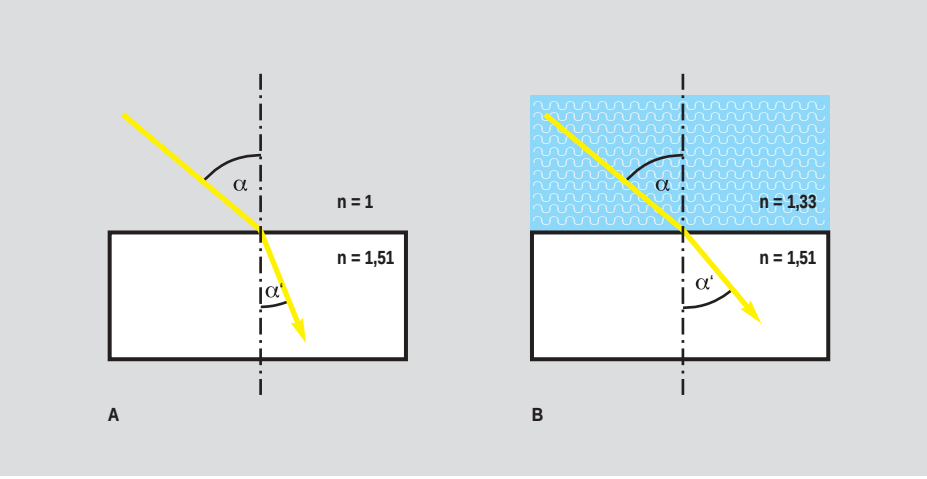
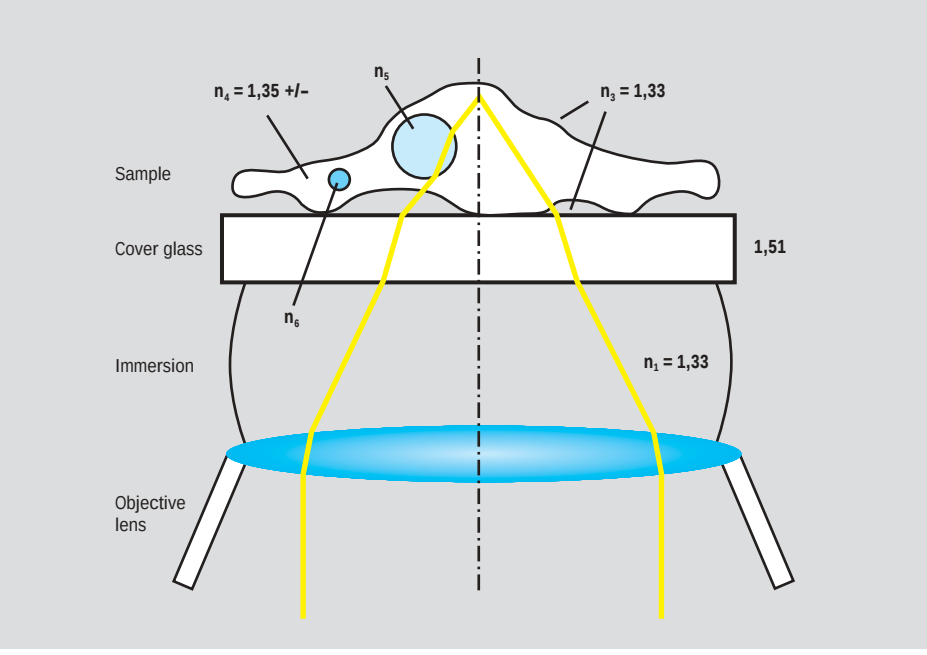


Fig. 2: Refractive index changes – **A)** Light ray from air into glass. A refractive index change occurs on the air-glass boundary leading to refraction of the light. The ray changes its direction and therefore the angle to the optical axis α changes to α'. **B)** Light ray from water into glass. The change of refractive index is less compared to the example described in A and correspondingly the angular change α to α' is less, too.



Optical systems, such as objective lenses for microscopes, are designed under the assumption, that the object (consisting of sample, cover slip and immersion medium) has a constant refractive index. Therefore, lenses for different immersion media are available. A lens, designed for water immersion is not suitable for samples embedded in resin, and an oil immersion lens will not give good results from living samples in buffer solution. If there is refractive index mismatch there are four main effects that cause errors and losses in resolution and intensity:

1. Due to different angles by which the light passes from the cover slip into the sample, the focus plane is in different positions for different refractive indices. If the immersion medium and the sample have identical indices, the optical path length is constant and therefore the focus position will change exactly in register with the mechanical focus changes. If the immersion medium and the sample have different indices, the optical path will vary during the focusing, causing the travel distances of the focal plane to differ from the mechanical focus. As a consequence, the sample will appear **elongated** or **squeezed along** the **z-axis** in three dimensional stack acquisitions (see Fig. 4b). This fault can be corrected for by simple rescaling of the z-axis in the data with the ratio of the refractive indices.

Fig. 3: Matching optical components – For optimal imaging conditions optical components (microscope objective lens, immersion medium, embedding medium of specimen) have to be matched. The example shown here is a specimen mounted in water/buffer (n = 1.33). A water lens is used with water immersion (n = 1.33). Inside the cell each compartment has a different refractive index, with diffraction effects occuring at the interfaces (see left light ray).

Note: A water lens will normally have a cover glass correction collar, to allow the lens to adapt to thickness variations.

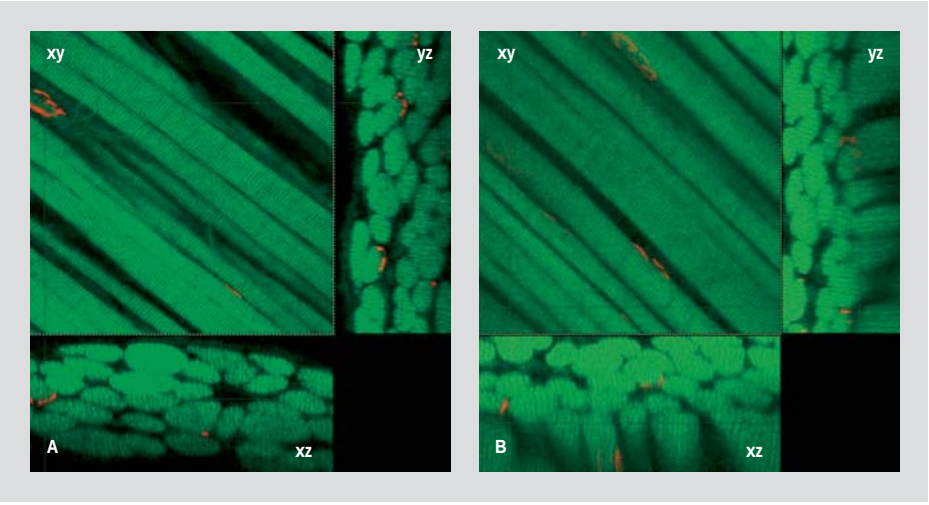


Fig. 4: Muscle tissue embedded in glycerol/water (80/20%), recorded stack: 100 µm.
For imaging of **A** a glycerol lens – for imaging of **B** an oil lens has been used. Note the spherical aberrations in xz- and yz-projection in **B** resulting from unmatched optical components (embedding medium, immersion medium and objective lens).
Courtesy of Dr. Günther Giese, MPI, Heidelberg

2. The refractive index is dependant upon wavelength. Values of refractive index indicated at media or glass-types refer in most cases to the yellow Na D-line. If the wrong media are used, the colour correction is affected because media have different dispersions (the relation of refractive index and wavelength). Chromatic errors will be obvious if mounting medium is not optimal for the specimen. In a multi-labeled fluorescent sample this may result in each channel having a **different axial (z) registration**, which will impair the coincidence of structures.
3. Using the wrong media will also cause strong spherical aberrations (see Fig. 4b) – the point is then not focused to a diffraction-limited point, but to a fuzzy cloud. The **optical resolution** is directly associated to the diameter of the diffraction-limited focus – and will be as **fuzzy** as the cloud – if refractive index is incorrect.
4. As a second consequence of spherical aberrations, the intensity will rapidly drop, when focusing into the sample. This will add **intensity loss** to absorption and diffraction effects, especially in thicker samples (> 10 µm). Thus, a precondition for deep penetration, also in 2-photon microscopy, is a perfect index match.

Perfect imaging by matching refractive index of glycerol embedding media and immersion medium

Objective lenses to adapt to different mounting and immersion media, are available: air ("dry lens"), water immersion with cover slip, water immersion without cover slip ("dipping lenses"), glycerol immersion, oil immersion and multiple immersion (only for low magnification).

Note: There are glycerol objectives specialized for far UV and quartz cover slips only.

In modern biology, common mounting media have refractive indices in the range of 1,45, corresponding to a glycerol/water mixture (80 / 20%). Ideally, the immersion should have equal properties (refractive index and dispersion), and the lens should be designed for this medium. To date there has not been a lens developed to match the refractive index of glycerol or glycerol/water and which may also be used with standard cover glasses.

These samples have traditionally been observed with oil immersion- or water immersion lenses; both of which will perform sub-optimally under these conditions. This is a major research limitation when thick samples in neuroscience (brain slices), developmental biology (whole larvae), histology or pathology are to be imaged.

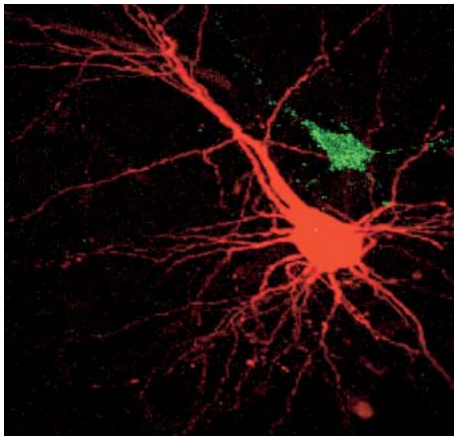


Fig. 5: Brain slice, pyramidal neurons filled by patch clamping embedded in glycerol/water (80/20%)
Maximum projection, xyz scan, z: 110 µm
Red: Alexa 594, Green: OEB-1
Courtesy of Dr. Thomas Nevian, MPI, Heidelberg.

In order to full fill the preconditions for best optical performance for imaging, Leica Microsystems has developed different types of objective lenses: Oil lenses for thin fixed specimens and water lenses for living specimens in aqueous solutions.

The recent innovation from Leica Microsystems is a new lens dedicated to the work with glycerol/water or similarly embedded specimen:

Leica HCX PL APO 63x/
1.3 GLYC CORR CS (21° C)

with apochromatic colour correction, maximum aperture and extra long working distance (280 µm).

Glycerol objective with correction collar: Easy adjustment for compensation of index changes

The new glycerol objective has a correction collar. Refractive index variation coming from changes of the composition of the mounting media, or from variations of cover glass thickness or temperature can now be corrected for. Assuming refractive index and cover glass thickness are optimal, the user can now compensate for small changes in temperature. Thus, the three parameters of refractive index – correction of the mounting medium, cover glass thickness, and temperature – can be adjusted by turning the correction collar:

Optimising the Objective for Refractive Index Change in the Specimen

Step 1: For correct adjustment the sample should be scanned in xz, using reflection mode (excitation laser line set above the detection window). Apply a glow-over LUT to help show areas of saturation within the image.

Step 2: The horizontal line coming from the reflection of the interface between the cover glass and the immersion medium should be visible. Normally, the reflection coming from the cover glass interface is brighter than reflection from the interface of the glass slide. It is this reflection line from the cover glass we use to fine-tune the objective.

Step 3: While scanning, turn the correction ring until the maximum intensity of the reflection line is visible, and then drops away. Next, slowly turn the ring in the opposite direction until the maximum intensity value is once again reached. The ring position where the line shows a maximum intensity is equal to the correct adjusted position for the sample (Fig. 6).

Note: One may also verify this procedure by observing changes in intensity of structures within the sample itself.

Glycerol objective with correction collar.

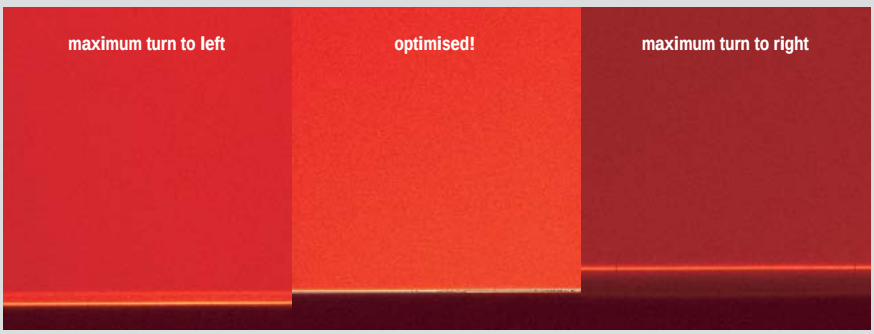


Fig. 6: xz-scan of 10 µM FITC in glycerol/water (80/20%) at 3 different positions of the correction collar. Adjustment is done according to the brightest reflection signal coming from the interface between cover glass and immersion medium. This reflection is seen as a bright line on the bottom of the images. The optimised position is shown in the middle.

Adaptation to the actual refractive index

The glycerol objective works optimal for refractive index ne = 1,451 for a mixture of 80% glycerol / 20% water. **Turning the correction ring can easily compensate index variations between 1,447 and 1,455.** This range of adjustment allows the observation of mixtures with glycerol or similar media like Vectashield in different concentrations of commonly used mounting media which has a refractive index similar to glycerol-water mixture of 80 / 20% (see Table 1). Table 2 demonstrates the changes in refractive indices with mediums of varying glycerol content.

Medium	Refractive Index
Water	1.333
100% PBS pH 8,9	1.3348
50% Vectashield + 50% PBS	1.39
50% PBS pH 8,9 + 50% Glycerol	1.4063
80% Glycerol/20% Water	1.451
100% Vectashield	1.4523
100% Glycerol	1.46
Moviol	1.46
50% Vectashield + 50% Glycerol	1.4634
Kaisers Glycerol Jelly	1.47
Oil	1.518
Canada Balm	1.5225
Glass	1.51

Table 1: Refractive index of different immersion and mounting media and glass.

Glycerol-Content	n
99.5	1.4750
90	1.4629
85	1.4572
80	1.4511
75	1.4447

Table 2: Refractive index (n) in relation to glycerol content.

Penetration depth: Comparison of glycerol and oil-objective

The z-response (penetration depth, signal intensity visible along the z-axis of the specimen) can be measured by the investigation of a test specimen. The recommended test specimen is prepared as follows:

FITC (fluoresceine-iso-thio-cyanat) is diluted in a glycerol/water (80/20%) mixture, concentration of FITC: 10 µM. A 100 µl aliquote of this solution is placed in a cavity slide, covered by a cover slip and sealed with nail polish.

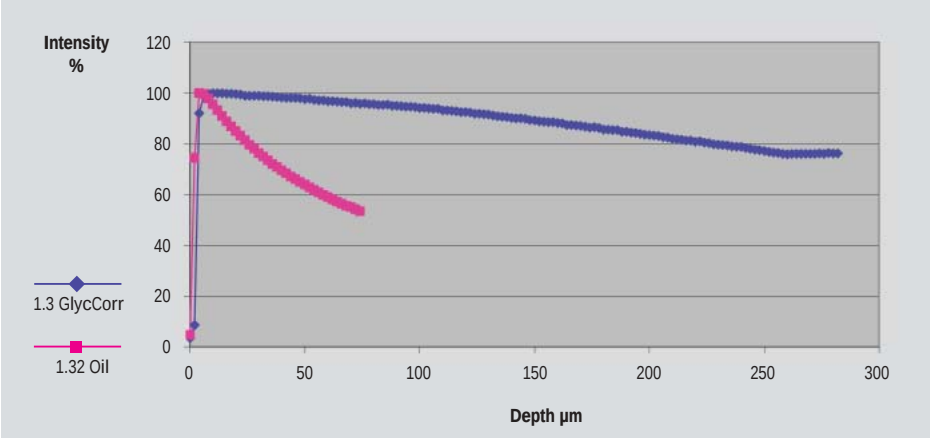


Fig. 7: Comparison of penetration depth of glycerol and oil objective.
Data obtained from xyz-series of 10 µM FITC in glycerol/water (80/20%) mounted on cavity slides. 1.3 GlycCorr = glycerol objective with numerical aperture of 1.3; 1.32 Oil = oil objective with numerical aperture of 1.32.
Note the slow drop in intensity observed for the use of the glycerol lens in comparison to the strong intensity drop observed for the use of the oil lens.

This specimen is then imaged at different focal planes along the z-axis (xyz-series) comparing the glycerol immersion objective and oil immersion objective. Fig. 7 shows the results.

At 50 µm depth the oil-objective shows a sharp intensity drop of 40% due to the spherical aberration effects. The glycerol objective on the other hand shows only a slow drop in intensity at increased penetration depth, to 10% at 150 µm. Moreover, the total loss of signal intensity at the maximum working distance (280 µm) is only 25% for the glycerol objective. This in fact may also be caused by absorption of light by the fluorochrome molecules. Using the oil objective the intensity quickly drops down to 50% at the maximum working distance (70 µm). Spherical aberration causes a loss of signal intensity due to an enlargement of the illuminated focus.

Extra-long free working distance at high numerical aperture

The 63x glycerol lens is designed for investigation of thick fixed specimen. It therefore has an extra long free working distance of 280 µm (see Fig. 7) with a high numerical aperture (1.3). A comparable oil lens with similar high numerical aperture would usually have a working distance of only 70 ... 100 µm.

Literature

- S. Hell, G. Reiner, C. Cremer & E.H.K. Stelzer. Aberrations in confocal fluorescence microscopy induced by mismatches in refractive index. Journal of Microscopy. Vol. 169, Pt 3, March 1993, pp. 391-405
- S. Hell & E.H.K. Stelzer. Lens aberrations in Confocal Fluorescence Microscopy. In: Handbook of Biological Confocal Microscopy, 1995. Edit. James B. Pawley, pp. 347-354
- N. Martini, J. Bewersdorf & S. Hell 2002. A new high-aperture glycerol immersion objective lens and its application to 3D-fluorescence microscopy. Journal of Microscopy, Vol. 206, Pt 2, May 2002, pp. 146-151

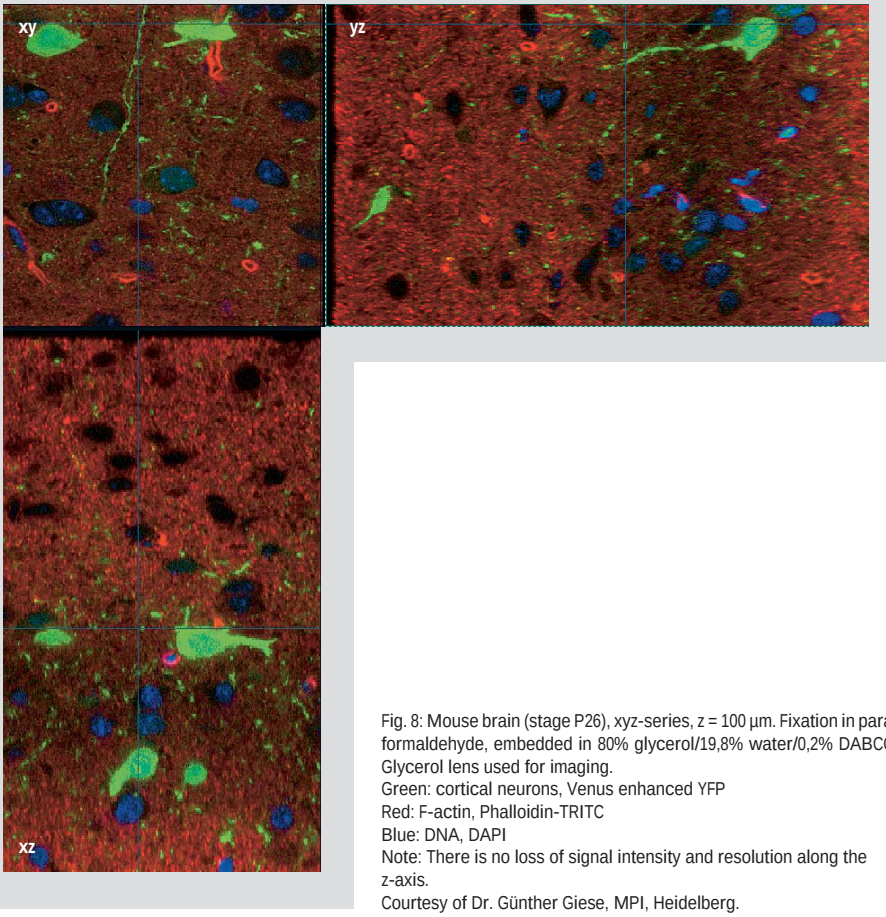


Fig. 8: Mouse brain (stage P26), xyz-series, z = 100 µm. Fixation in paraformaldehyde, embedded in 80% glycerol/19,8% water/0,2% DABCO. Glycerol lens used for imaging.
Green: cortical neurons, Venus enhanced YFP
Red: F-actin, Phalloidin-TRITC
Blue: DNA, DAPI
Note: There is no loss of signal intensity and resolution along the z-axis.
Courtesy of Dr. Günther Giese, MPI, Heidelberg.

Leica Microsystems – the brand for outstanding products

Leica Microsystems' mission is to be the world's first-choice provider of innovative solutions to our customers' needs for vision, measurement, lithography and analysis of microstructures.

Leica, the leading brand for microscopes and scientific instruments, developed from five brand names, all with a long tradition: Wild, Leitz, Reichert, Jung and Cambridge Instruments. Yet Leica symbolizes innovation as well as tradition.

Leica Microsystems – an international company with a strong network of customer services

Australia:	Gladesville	Tel. +61 2 9879 9700	Fax +61 2 9817 8358
Austria:	Vienna	Tel. +43 1 486 80 50 0	Fax +43 1 486 80 50 30
Canada:	Richmond Hill/Ontario	Tel. +1 905 762 2000	Fax +1 905 762 8937
Denmark:	Herlev	Tel. +45 4454 0101	Fax +45 4454 0111
France:	Rueil-Malmaison	Tel. +33 1 473 285 85	Fax +33 1 473 285 86
Germany:	Bensheim	Tel. +49 6251 136 0	Fax +49 6251 136 155
Italy:	Milan	Tel. +39 0257 486.1	Fax +39 0257 40 3273
Japan:	Tokyo	Tel. +81 3 5435 9600	Fax +81 3 5435 9615
Korea:	Seoul	Tel. +82 2 514 65 43	Fax +82 2 514 65 48
Netherlands:	Rijswijk	Tel. +31 70 4132 100	Fax +31 70 4132 109
People's Rep. of China:	Hong Kong	Tel. +852 2564 6699	Fax +852 2564 4163
Portugal:	Lisbon	Tel. +351 21 388 9112	Fax +351 21 385 4668
Singapore		Tel. +65 6779 7823	Fax +65 6773 0628
Spain:	Barcelona	Tel. +34 93 494 95 30	Fax +34 93 494 95 32
Sweden:	Sollentuna	Tel. +46 8 625 45 45	Fax +46 8 625 45 10
Switzerland:	Glattbrugg	Tel. +41 1 809 34 34	Fax +41 1 809 34 44
United Kingdom:	Milton Keynes	Tel. +44 1908 246 246	Fax +44 1908 609 992
USA:	Bannockburn/Illinois	Tel. +1 847 405 0123	Fax +1 847 405 0164

and representatives of Leica Microsystems in more than 100 countries.

The companies of the Leica Microsystems Group operate internationally in four business segments, where we rank with the market leaders.

Microscopy Systems

Our expertise in microscopy is the basis for all our solutions for visualization, measurement and analysis of microstructures in life sciences and industry. With confocal laser technology and image analysis systems, we provide three-dimensional viewing facilities and offer new solutions for cytogenetics, pathology and materials sciences.

Specimen Preparation

We provide comprehensive systems and services for clinical histo- and cytopathology applications, biomedical research and industrial quality assurance. Our product range includes instruments, systems and consumables for tissue infiltration and embedding, microtomes and cryostats as well as automated stainers and coverslippers.

Medical Equipment

Innovative technologies in our surgical microscopes offer new therapeutic approaches in microsurgery.

Semiconductor Equipment

Our automated, leading-edge measurement and inspection systems and our E-beam lithography systems make us the first choice supplier for semiconductor manufacturers all over the world.

@ www.confocal-microscopy.com

Leica MICROSYSTEMS