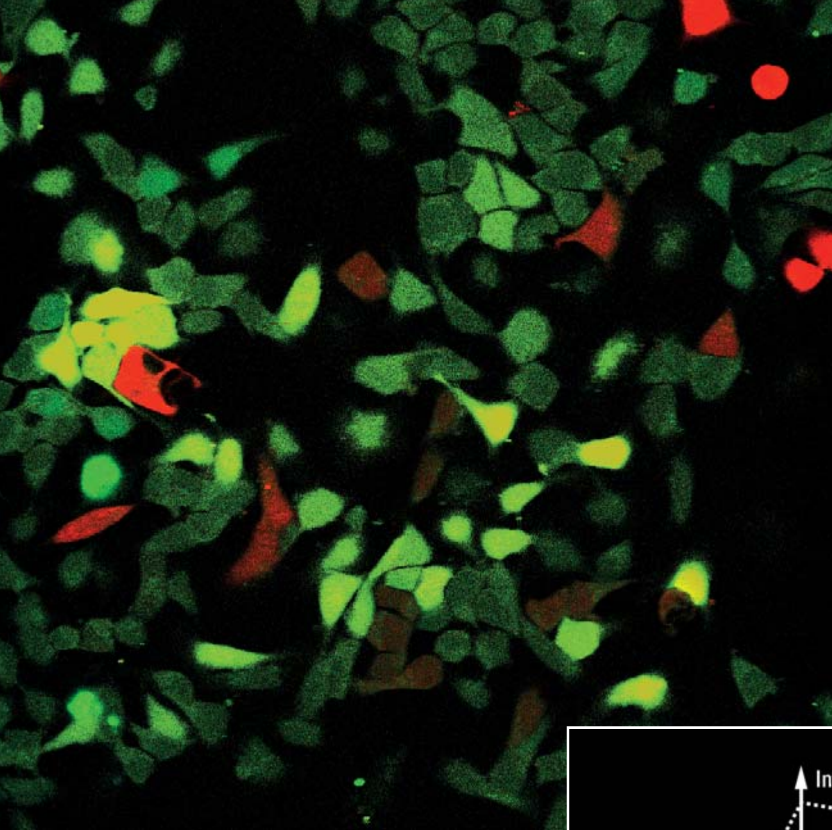
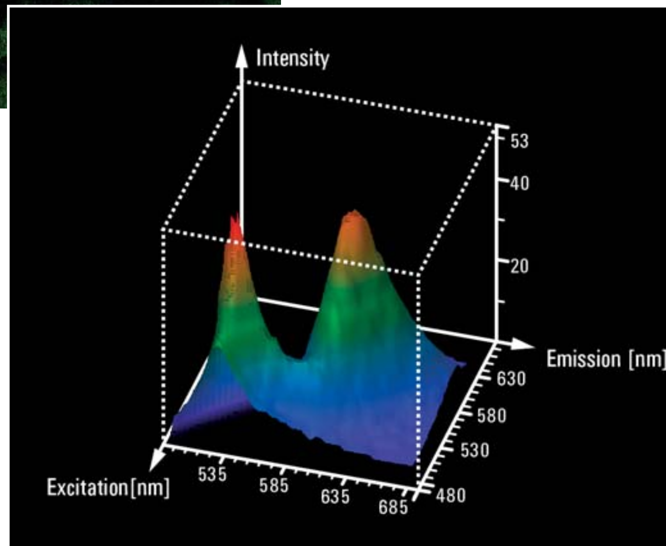


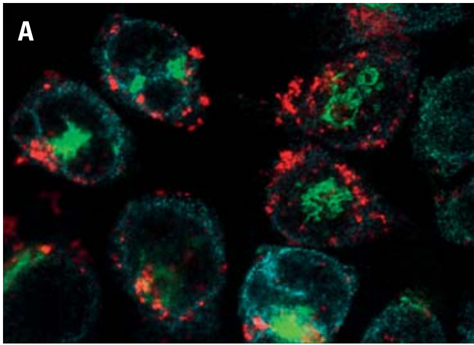


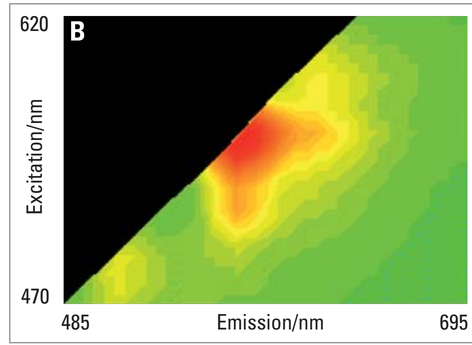
Lambda Square Mapping and FLIM

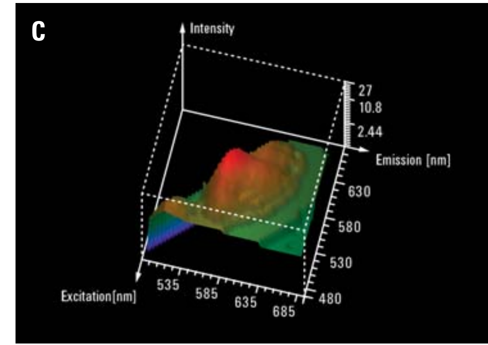
Explore Photonic Landscapes with the Leica TCS SP5 X

Living up to Life

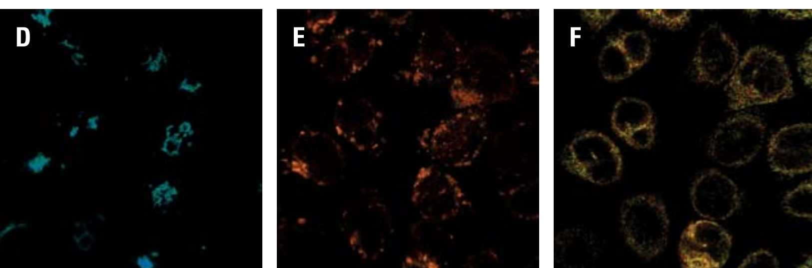
Leica
MICROSYSTEMS







- Full spectral analysis of images and lifetime measurements
- User guidance by interactive experiment definition
- Optimization of excitation and detection range
- Complete characterization of natural fluorescence
- “Dye Hunting”: Screen for fluorescent proteins and discover new dyes



Laser sources in microscopy

In the past the design of confocal microscopes was linked to lasers as light sources. Only lasers generate beams that are intense and at the same time easy to focus. The advantage of single, spectrally narrow laser lines of classical lasers is also their biggest limitation: Biological samples often contain various spectrally different fluorochromes. Their visualization require systems that contain a series of differently emitting lasers. The design, control and maintenance of such devices is challenging.

Leica TCS SP5 X

New Applications!

White light source for confocal

A "white" light laser which emits a continuous spectrum solves these problems. The Leica TCS SP5 X uses such a white light source that emits from 470 to 670 nanometers and still provides beam properties of a conventional laser. In conjunction with an acousto-optical tunable filter (AOTF), it offers stepless tunable excitation wavelengths over the full range. Up to 8 of such lines may simultaneously be picked and each color be individually attenuated. This allows optimized excitation of any dye in the visible range.

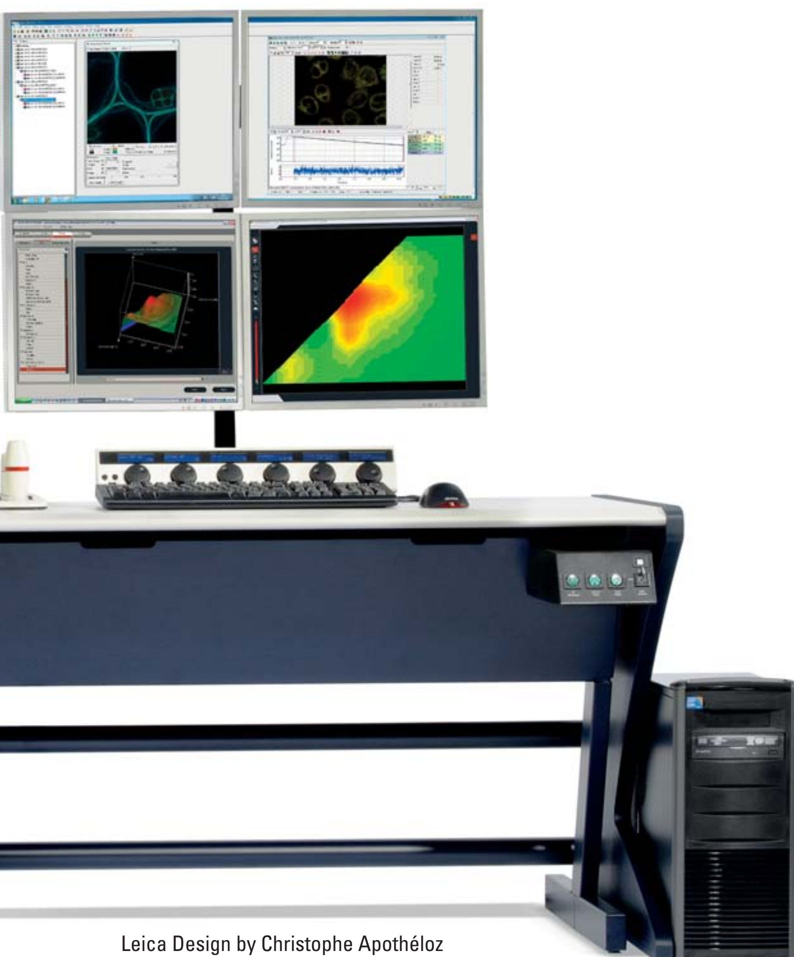
Lambda square fluorescence mapping

Excitation spectra can be acquired for any defined emission band of the Leica SP Detector. A two-dimensional spectrum can now be reconstructed from a series of excitation spectra. The resulting Lambda Square (λ^2) landscape discriminates fluorophores with identical emission or excitation, which is essential for multi-color fluorescence experiments.

Tunable excitation for FLIM

The White Light Laser (WLL) is a pulsed light source. Therefore, it is also applicable for **F**luorescence **L**ifetime **IM**aging (FLIM). This adds a completely new dimension to the experimental tool box: correlation of excitation wavelength, emission wavelength (SP FLIM), and fluorescence lifetime. This method solves problems where dyes in use have identical excitation and emission properties. The molecule species can now be separated by their lifetimes. With the Leica TCS SP5 X, new photonic landscapes await your exploration!

Sample A to F: Fixed cells with triple staining: GalNacT2_GFP, LAMP-546, Calnexin 594. (A) Intensity image, (B,C) Logarithmic scaled λ^2 plot. (D,E,F) Average fluorescence lifetime using excitation at 486 nm, 542 nm, or 594 nm. Courtesy of Dr. Matthias Weiss, Cellular Biophysics Group, Bioquant, Heidelberg, Germany.



Leica Design by Christophe Apothéloz

Lambda Square Mapping

"Great tool for full spectral characterization of new preparations"

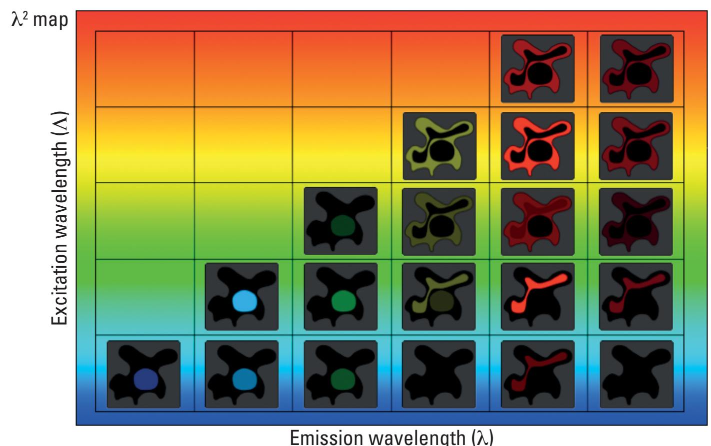
Prof. Kees Jalink, Department of Cell Biology, The Netherlands Cancer Institute Amsterdam, The Netherlands

Benefits of λ^2 maps

- Obtain full spectral information in each pixel of the image
- Understand samples with very complex fluorescence characteristics
- Depict multifaceted features in one view
- Obtain precise spectral information by the excitation and detection range positioning of 1 nm

Lambda Square Mapping relates the fluorescence intensity to both the excitation and the emission wavelength. For each position in one λ dimension, e.g., the emission wavelength (λ), a full series of intensity measurements is acquired for the other λ dimension, e.g., the excitation wavelength (Λ). In essence, an excitation spectrum is recorded for any emission wavelength. The measured data for each excitation emission pair is displayed as full images.

The result is a two-dimensional map that plots the sample's brightness as a function of excitation and emission. In general, each recorded pixel contains such a full photonic landscape. To enhance signal-to-noise ratio it is convenient to select regions of interest. Finally, the λ^2 map sums up all values for these regions and displays the distribution of fluorescence depending on excitation and emission color.



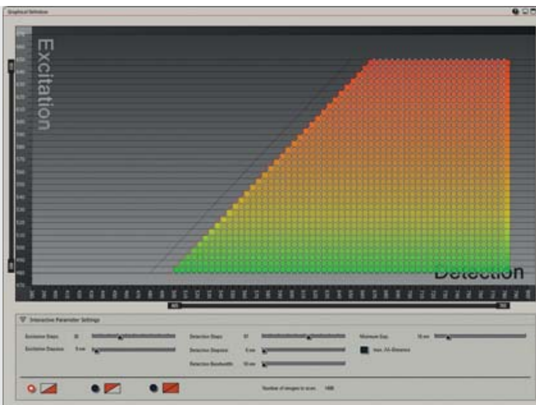
Why is this beneficial?

Modern research in biology typically targets samples with multiple fluorochromes. Also, intrinsic fluorescence of samples, e.g., biofilms, shows a complex composition of fluorescent species. A single emission spectrum would be inadequate for distinguishing between these fluorochromes; in many cases, a peak would be composed of two different contributions. These contributions can now be separated using the λ^2 map. Here, two peaks having similar emission but different excitation properties are displayed.

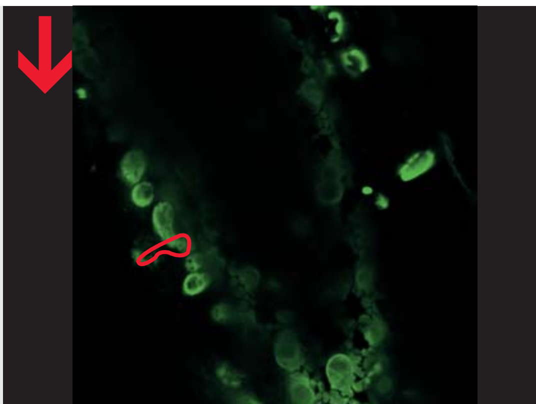
In general, λ^2 mapping is a very convenient tool to characterize the fluorescence of any sample. This is also important for well-known fluorochromes, as their spectral characteristics may depend significantly on the environmental conditions of the molecule.

Conducting Lambda Square Mapping

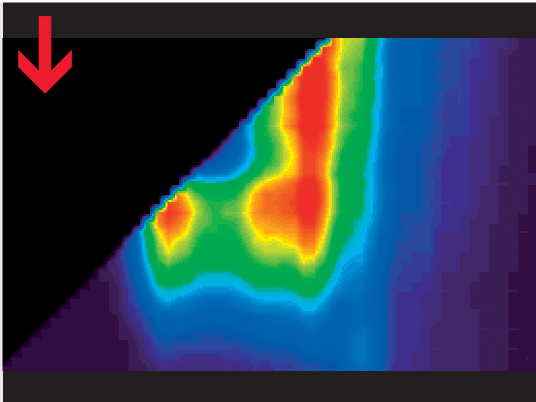
Step 1:
Define the λ^2 scan and acquire the corresponding data collection.



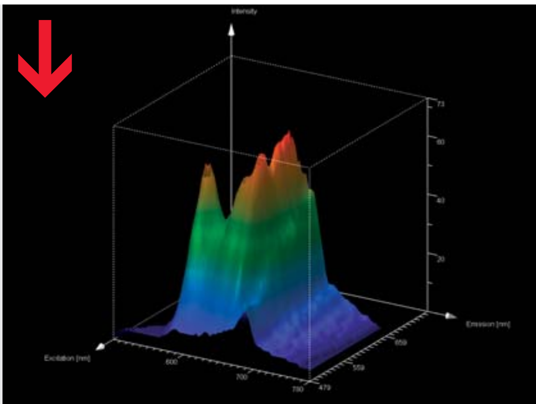
Step 2:
Select regions of interest on the data collection.



Step 3:
Calculate the λ^2 contour plot.



Step 4:
Calculate a three dimensional display of intensity over λ^2.



Never Lost in Photonic Space

Applications of λ^2 mapping

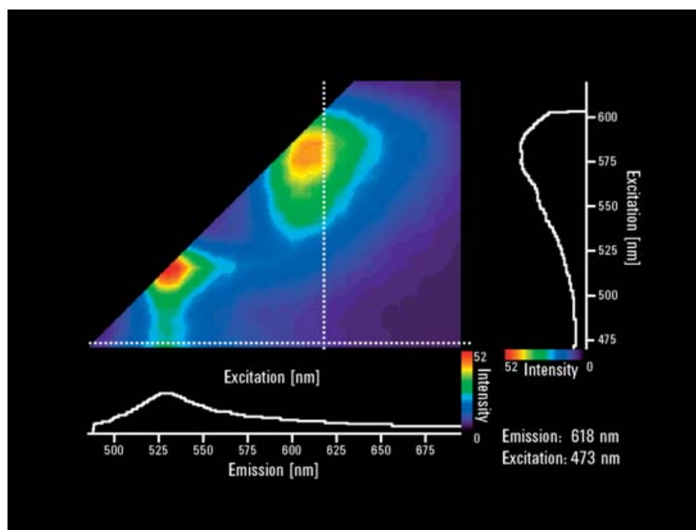
- Characterization of intrinsic fluorescence
- Finding the optimal staining for autofluorescent samples
- Dye identification and localization in cells and structures
- Characterization of local environment of fluorophores
- Screening for fluorescent proteins
- Development and characterization of new fluorescent markers
- Optimization of instrument settings for multiple stained samples

User guidance by graphical experiment definition

For the design of a λ^2 experiment, many interrelated parameters need to be defined. An intelligent, interactive graphical user interface supports the user during experiment definition: the GUI displays the defined data array immediately. If any parameter is changed, effects on the final data array are shown. This facilitates experimental setup and reduces the possibility of wrong experimental definitions.

Easy navigation on data collections

After data acquisition, the user may want to closely inspect the obtained images. Navigation on the related data collection is easy: select a certain position in the λ^2 contour plot by moving the crosshair, and the corresponding image will be displayed.



Obtain information fast!

Sophisticated data evaluation tools give a fast overview and depict the full spectral information at a glance.

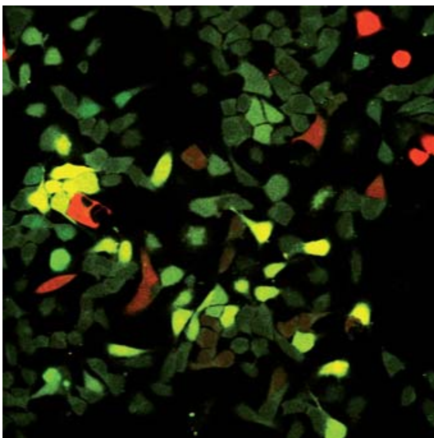
2D Plot: This plot reveals characteristic excitation emission patterns for fast, easy recognition of fluorescent molecules. The displayed intensity is color-coded. The underlying look-up table can be scaled and optimized to the data by the user.

Spectral sectioning tool: One-dimensional excitation and emission spectra along the axes of the movable crosshair are displayed. This allows easy comparison with already known excitation or emission spectra.

3D Plot: This plot displays intensity values as color and height. Discover hidden information by free rotation of the plot.

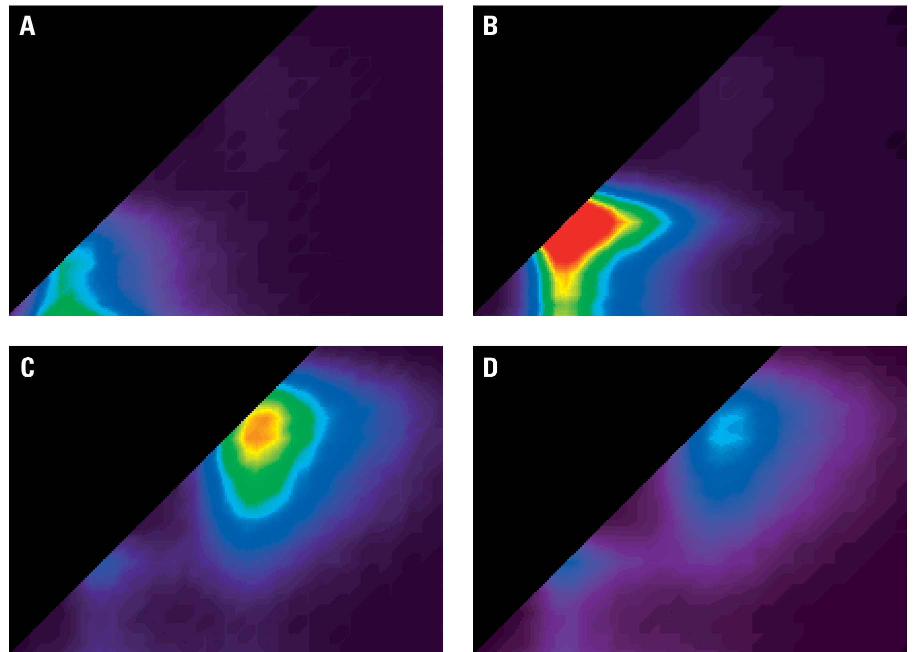
Optimize Your Experiment for Best Data Quality

The λ² **contour plot** reveals the different excitation-emission peaks of multiple stained samples. This information can be used to optimize instrument settings, resulting in minimized cross excitation and crosstalk combined with maximum excitation and detection efficiency. This artifact reduction enhances the information content of images. In addition, the spectral optimization of excitation and detection reduces acquisition time and photobleaching.



Overview image:
Mixture of fixed cells expressing 4 different fluorescent proteins (FP).

λ² contour plots of single cells expressing different FPs.

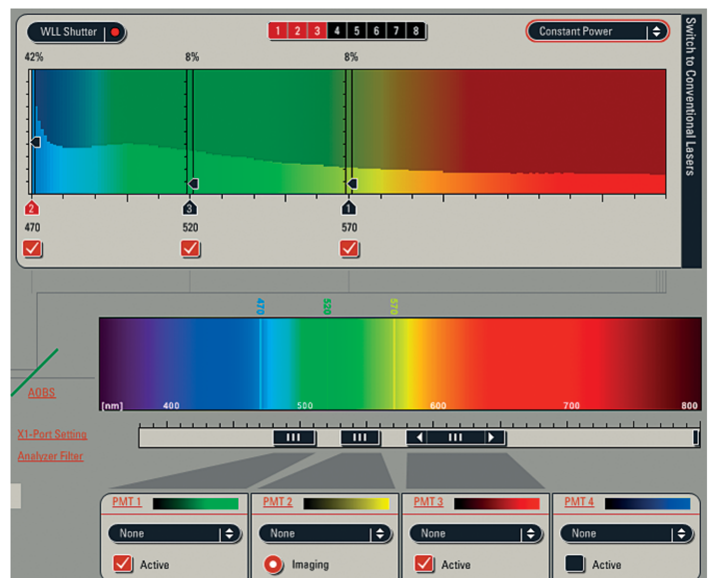


Scaling of λ² plots:
Excitation range: 470 to 620 nm
Detection range: 480 to 700 nm

The different FPs can be identified by their characteristic spectrum. Excitation emission peaks are given in brackets. All cells show a small autofluorescence peak at (512, 533) nm.
A – GFP (470, 511)
B – YFP (515, 530)
C – mCherry (577, 605)
D – mKATE (582, 620)

According to the λ² contour plots, instrument parameter settings are optimized. The different FPs can now be identified within one image. The fluorescence intensity of mKATE transfected cells is much lower compared to cells labeled with mCherry.

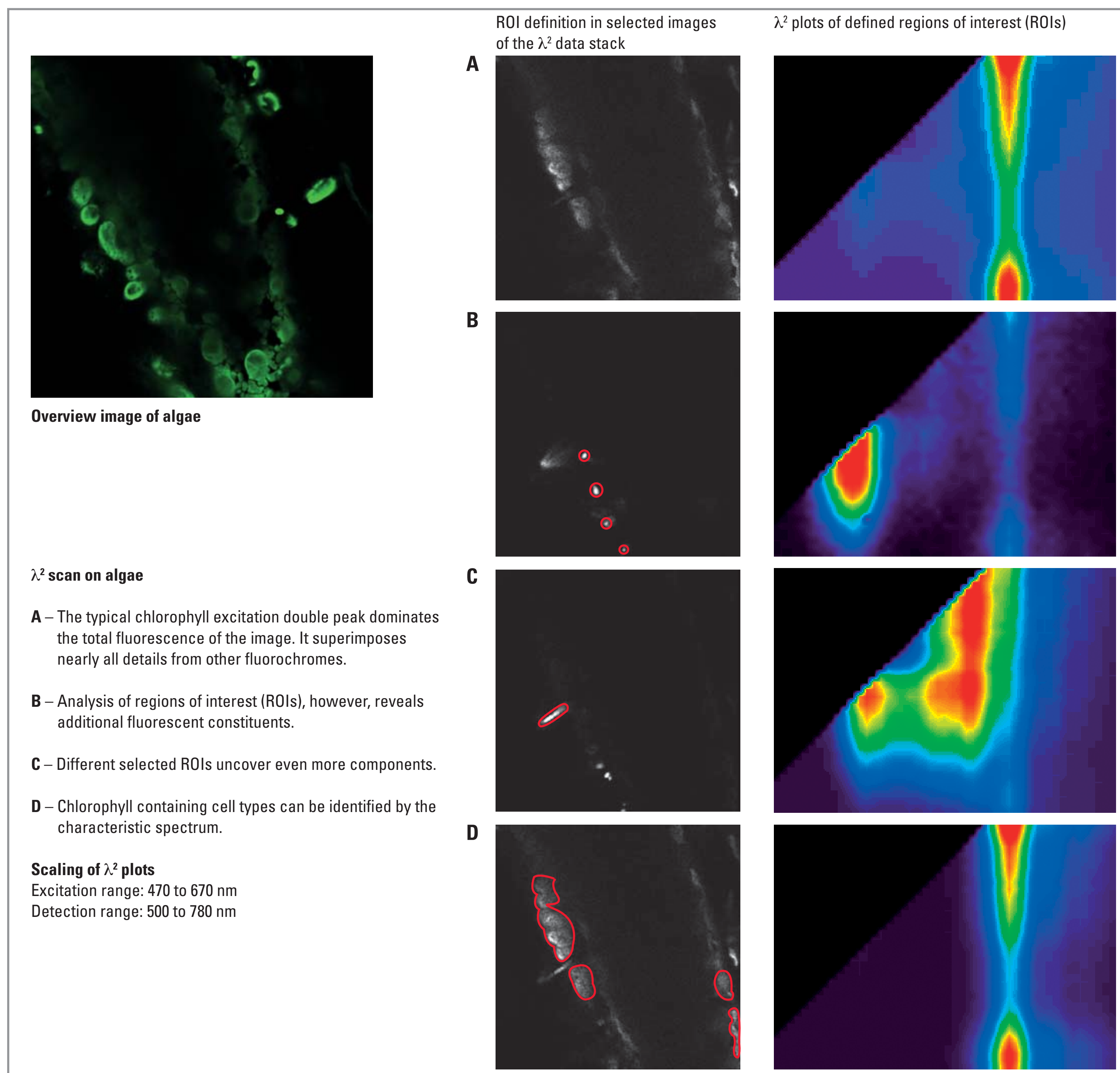
Sample: courtesy of Prof. Kees Jalink, Department of Cell Biology, The Netherlands Cancer Institute Amsterdam, The Netherlands



Explore Natural Fluorescence

Display previously invisible contributions of minor populations

Many biological structures contain fluorescent components that can be used for imaging without the need for external labels. The autofluorescence can provide valuable information about the composition of functional molecular complexes or substances that characterize the metabolic state of cells or tissues. The λ^2 scan and its analysis tools extract and visualize this information in an easy way. λ^2 maps of selected regions of interest display contributions of minor populations, that were not visible before.



Overview image of algae

λ^2 scan on algae

A – The typical chlorophyll excitation double peak dominates the total fluorescence of the image. It superimposes nearly all details from other fluorochromes.

B – Analysis of regions of interest (ROIs), however, reveals additional fluorescent constituents.

C – Different selected ROIs uncover even more components.

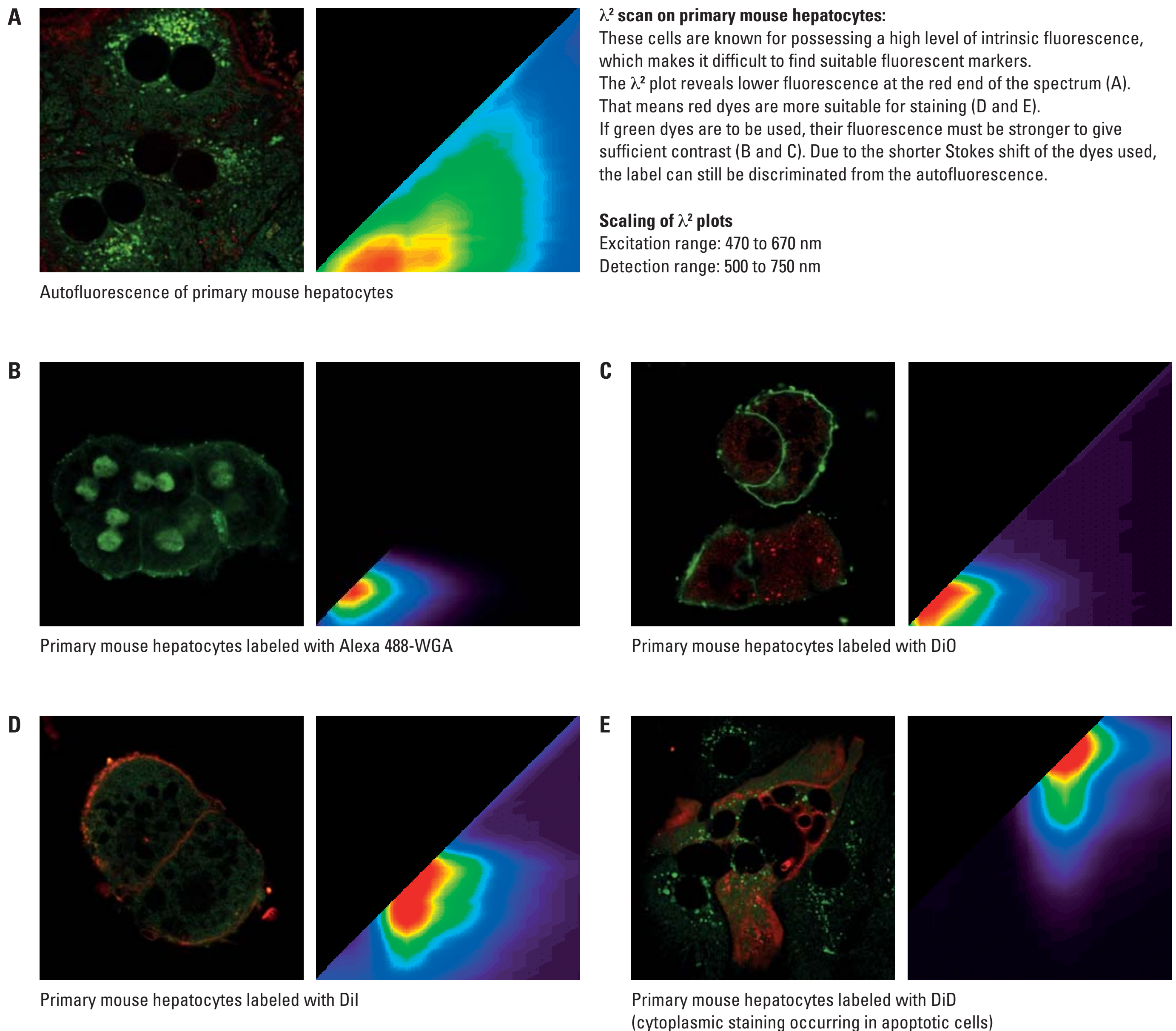
D – Chlorophyll containing cell types can be identified by the characteristic spectrum.

Scaling of λ^2 plots
Excitation range: 470 to 670 nm
Detection range: 500 to 780 nm

Find the Best Fluorescent Label

Achieve the highest image contrast with the optimal staining

Fluorescent labeling is a common technique for visualizing non-colored structures. However, intrinsic fluorescence may overlay or even hide the signal from the artificial label. To evaluate the impact of the autofluorescence on the final image and to reduce its contribution, it is practical to first measure the full spectrum of the sample itself. This information helps to search for fluorescent markers that are excited and emit in a range of low autofluorescence. Thus the optimal staining that yields the highest image contrast can be found.



λ^2 scan on primary mouse hepatocytes:
These cells are known for possessing a high level of intrinsic fluorescence, which makes it difficult to find suitable fluorescent markers.
The λ^2 plot reveals lower fluorescence at the red end of the spectrum (A). That means red dyes are more suitable for staining (D and E).
If green dyes are to be used, their fluorescence must be stronger to give sufficient contrast (B and C). Due to the shorter Stokes shift of the dyes used, the label can still be discriminated from the autofluorescence.

Scaling of λ^2 plots
Excitation range: 470 to 670 nm
Detection range: 500 to 750 nm

A: Autofluorescence of primary mouse hepatocytes

B: Primary mouse hepatocytes labeled with Alexa 488-WGA

C: Primary mouse hepatocytes labeled with DiO

D: Primary mouse hepatocytes labeled with DiI

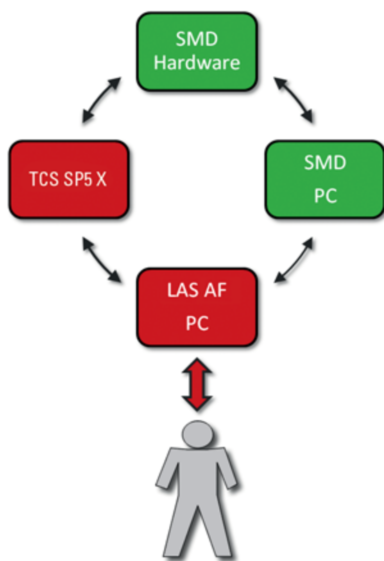
E: Primary mouse hepatocytes labeled with DiD (cytoplasmic staining occurring in apoptotic cells)

Sample: courtesy of Dr. René Meyer, Klingmüller Group, Systems Biology of Signal Transduction, DKFZ, Heidelberg, Germany

Combine the Power of FLIM and Leica TCS SP5 X

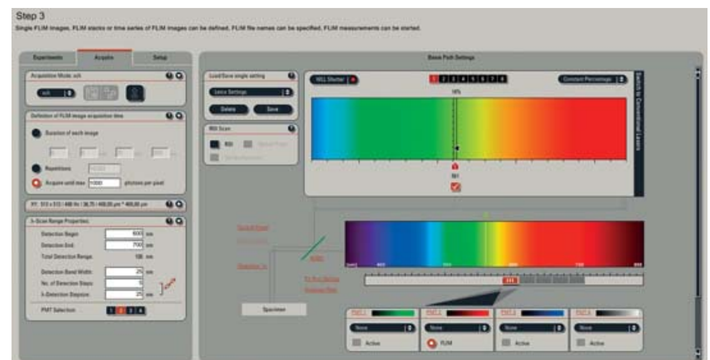
FLIM applications

- FLIM with orange and red dyes or FPs
- Full characterization of intrinsic fluorescence
- Dye identification
- Screening for fluorescent proteins
- Structure identification
- Molecular binding studies using FLIM-FRET with variable FRET pairs
- Development of new dyes

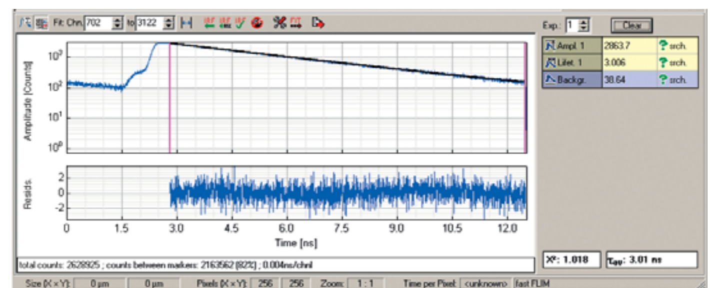


The Leica TCS SMD FLIM is a powerful system for fluorescence lifetime measurements and analysis. The seamless integration of TCSPC equipment from PicoQuant (Berlin, Germany) into the Leica TCS SP5 together with the intuitive application wizards in Leica LAS AF guarantee easy operation and high data quality.

The application range of the Leica TCS SMD FLIM is now expanded by the versatility of the White Light Laser (WLL): Being a pulsed laser, the WLL is used as a FLIM excitation source and is therefore integrated into the SMD FLIM wizard. This way the user can take advantage of both the convenience of the wizard and the flexibility of the WLL. The setup will cover FLIM applications in the μM range.



Leica LAS AF user interface of an Leica TCS SP5 X system with TCS SMD FLIM. A FLIM lambda detection scan is defined within the SMD FLIM wizard.



Fluorescence decay curve of T-Epac-W labeled with mTurquoise expressed in cells. Excitation was at 470 nm. The strictly monoexponential decay curve makes it an ideal donor in FRET experiments. FRET partner can be Venus.

Sample: courtesy of Prof. Kees Jalink, Department of Cell Biology, The Netherlands Cancer Institute Amsterdam, The Netherlands

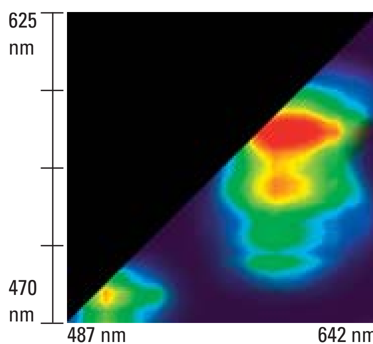
Get the Full Picture – Connecting Spectral and Lifetime Information

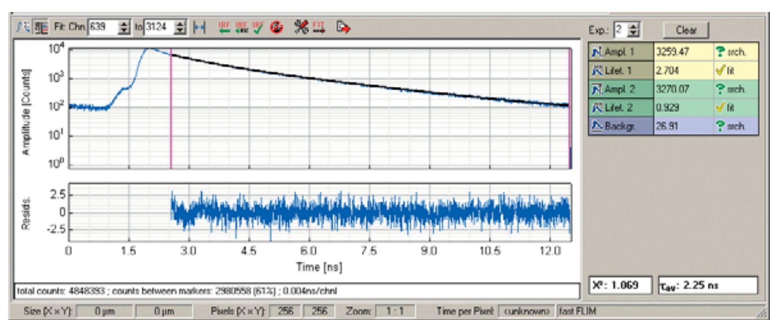
The use of λ^2 mapping and lifetime imaging extracts maximum information out of fluorescence data. This gives the user the highest flexibility and freedom in experimental design and analysis: dyes with identical spectral properties can be distinguishable by different fluorescence lifetimes; dyes with similar lifetimes can differ in their spectral characteristics. The comparison of spectral and lifetime information also enhances the reliability of data interpretation. For instance, in binding studies based on the FRET effect, analysis of spectral data is often used to validate results obtained with the FLIM approach and vice versa.

dTomato, mCherry, and mRFP are red fluorescent proteins that are commonly used for staining in living cells.
Using the possibilities of the WLL, the proteins can be discriminated, either by the λ^2 contour plot or by lifetime measurements.

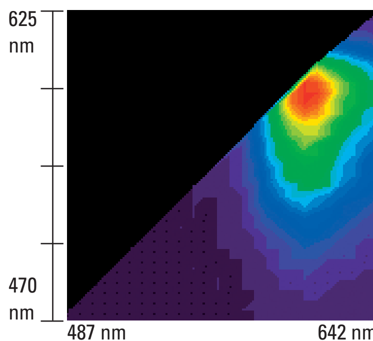
dTomato:
The λ^2 plot reveals two peaks reflecting the green-red maturation of the protein.

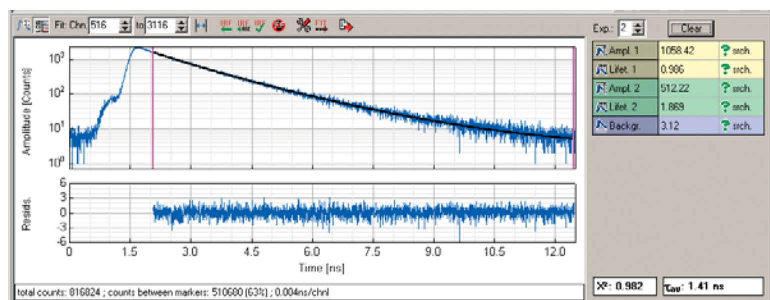
The lifetime is bi-exponential and longer compared to mCherry and mRFP.



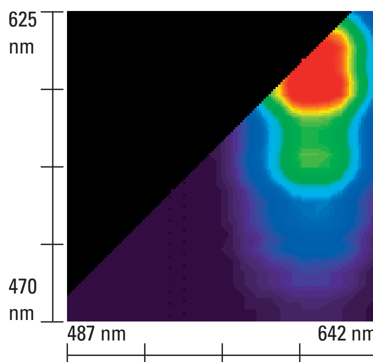


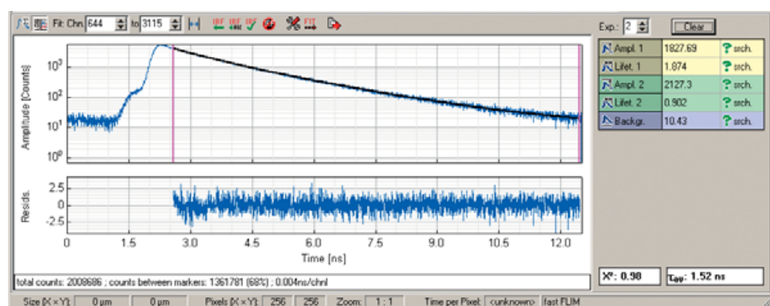
mCherry:
Only one peak is visible in the λ^2 plot. The lifetime is bi-exponential.





mRFP:
Although fluorescence decay of mRFP and mCherry are very similar, λ^2 plots differ, and enable the two proteins to be distinguished.





Sample: courtesy of Prof. Kees Jalink, Department of Cell Biology, The Netherlands Cancer Institute Amsterdam, The Netherlands

Further reading

1 R. Borlinghaus, H. Gugel, P. Albertano and V. Seyfried
Closing the spectral gap – the transition from fixed-parameter fluorescence to tunable devices in confocal microscopy. Proc. SPIE 6090 (2006)
2 A. Diaspro, K. Jalink, V. Caorsi, P. Bianchini: Solving the Challenge of Fluorescence, Leica TCS SP5 X: The first completely tunable confocal system.
reSOLUTION – Leica Confocal Application Letter 29 (2008)

“With the user, for the user” Leica Microsystems

Leica Microsystems operates globally in four divisions, where we rank with the market leaders.

• Life Science Division

The Leica Microsystems Life Science Division supports the imaging needs of the scientific community with advanced innovation and technical expertise for the visualization, measurement, and analysis of microstructures. Our strong focus on understanding scientific applications puts Leica Microsystems’ customers at the leading edge of science.

• Industry Division

The Leica Microsystems Industry Division’s focus is to support customers’ pursuit of the highest quality end result. Leica Microsystems provide the best and most innovative imaging systems to see, measure, and analyze the microstructures in routine and research industrial applications, materials science, quality control, forensic science investigation, and educational applications.

• Biosystems Division

The Leica Microsystems Biosystems Division brings histopathology labs and researchers the highest-quality, most comprehensive product range. From patient to pathologist, the range includes the ideal product for each histology step and high-productivity workflow solutions for the entire lab. With complete histology systems featuring innovative automation and Novocastra™ reagents, Leica Microsystems creates better patient care through rapid turnaround, diagnostic confidence, and close customer collaboration.

• Medical Division

The Leica Microsystems Medical Division’s focus is to partner with and support surgeons and their care of patients with the highest-quality, most innovative surgical microscope technology today and into the future.

The statement by Ernst Leitz in 1907, “with the user, for the user,” describes the fruitful collaboration with end users and driving force of innovation at Leica Microsystems. We have developed five brand values to live up to this tradition: Pioneering, High-end Quality, Team Spirit, Dedication to Science, and Continuous Improvement. For us, living up to these values means: **Living up to Life**.

Active worldwide

Australia:	North Ryde	Tel. +61 2 8870 3500	Fax +61 2 9878 1055
Austria:	Vienna	Tel. +43 1 486 80 50 0	Fax +43 1 486 80 50 30
Belgium:	Groot Bijgaarden	Tel. +32 2 790 98 50	Fax +32 2 790 98 68
Canada:	Richmond Hill/Ontario	Tel. +1 905 762 2000	Fax +1 905 762 8937
Denmark:	Ballerup	Tel. +45 4454 0101	Fax +45 4454 0111
France:	Nanterre Cedex	Tel. +33 811 000 664	Fax +33 1 56 05 23 23
Germany:	Wetzlar	Tel. +49 64 41 29 40 00	Fax +49 64 41 29 41 55
Italy:	Milan	Tel. +39 02 574 861	Fax +39 02 574 03392
Japan:	Tokyo	Tel. +81 3 5421 2800	Fax +81 3 5421 2896
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:	Kista	Tel. +46 8 625 45 45	Fax +46 8 625 45 10
Switzerland:	Heerbrugg	Tel. +41 71 726 34 34	Fax +41 71 726 34 44
United Kingdom:	Milton Keynes	Tel. +44 800 298 2344	Fax +44 1908 246312
USA:	Buffalo Grove/Illinois	Tel. +1 847 405 0123	Fax +1 847 405 0164

and representatives in more than 100 countries

Order no.: **English 1593002013** • III/11/DX/DB.H. • Copyright © by Leica Microsystems CMS GmbH, Mannheim, Germany, 2011
LEICA and the Leica Logo are registered trademarks of Leica Microsystems IR GmbH.

www.leica-microsystems.com

Leica
MICROSYSTEMS