

Laser Microdissection Aids Studies in Alzheimer's Disease Identification of Candidate Genes

In recent years, laser microdissection has increasingly become established in the biological sciences as a means of acquiring homogeneous specimen material. To better understand the cellular and molecular basis of Alzheimer's disease, Dr. Thomas Deller (Professor of Anatomy and Director of the Institute of Clinical Neuroanatomy, Johann Wolfgang Goethe-Universität in Frankfurt, Germany) and his work group study regulatory molecules in the brains of mice after acute and chronic injury, as well as those in the brains of mice with Alzheimer's. The work group uses a laser microdissection system and combines this method with quantitative RT-PCR. Unlike traditional methods, this allows gene expression changes to be quantified on a cellular level.

Neurodegenerative diseases such as Alzheimer's disease lead to the loss of synapses and neurons in the CNS. The brain reacts to these changes with complex regulation mechanisms, known as lesion induced neuronal plasticity. An important process in this regard is the growth of new nerve fibers from surviving cells. This axonal sprouting results in partial reinnervation and new synapses and may contribute to a partial restoration of function. In Alzheimer's disease, however, there are indications that axonal sprouting can also take on an aberrant form. In the immediate vicinity of the amyloid plaques that characterize this disease, pathological sprouting processes have been observed which could worsen cognitive

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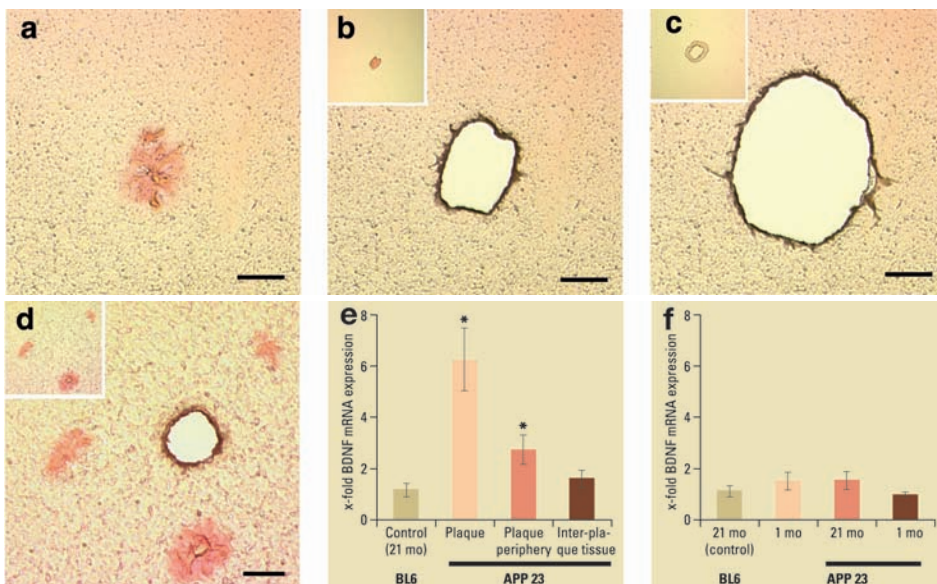


Fig. 1: Increasing BDNF mRNA expression in the direction of amyloid plaques. Cortex tissue of 21-month-old APP23 transgenic mice stained with Congo Red. **a:** Plaques in APP23 mice. **b:** Dissection of the plaques; **c:** Dissection of a 30µm wide ring of tissue. **d:** Dissection of tissue between the plaques. **e:** Results of the qPCR showed increasing BDNF mRNA expression in the direction of the plaques. **f:** Comparatively low BDNF mRNA expression in the tissue between the plaques. P 0.05; size of scale bar = 50µm in a–d.

The Objective

In this section, we like to highlight application-oriented stories of value to our readers.

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Visit the Leica Microsystems website to view a webcast from **Dr. Jennifer Lippincott-Schwartz** (NIH) entitled, "New Methodologies for Imaging Protein Transport, Turnover and Tology in Living Cells."

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Laser Microdissection

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deficits associated with Alzheimer's disease. If the effort to explain the functional significance of axonal sprouting, identify regulatory molecules, and ultimately intervene in the sprouting processes succeeds, new therapeutic intervention strategies may be developed for treating neurodegenerative diseases and acute brain injuries.

Neuronal plasticity after injury

Deller and his work group are particularly involved in researching the cellular and molecular basis of neuronal plasticity after brain lesion. Their focus is on the molecular regulation of axonal sprouting and its significance for Alzheimer's disease. To analyze the changes in the brain, APP23 transgenic mice were used, which, after six months, develop plaques in the brain that are similar to those caused by Alzheimer's disease [1].

Research focuses on astrocyte response.

One of Deller's research approaches is concerned with the response of the astrocytes [2], which play an important role in how the brain responds to an injury. These cells multiply in the affected brain regions and change their gene expression, particularly that of the GFAP (glial fibrillary acidic protein) molecule. The expression of GFAP mRNA is determined by the quantitative reverse transcriptase polymerase chain reaction (qPCR). Earlier studies with brain tissue, however, used tissue homogenates that contained various cell types. This meant that the mRNA could not be definitively traced to one source. Therefore, Deller's workgroup uses laser microdissection, which allows the isolation of defined cell populations.

Using the Leica Microsystems laser microdissection system, small areas can be isolated from a tissue segment. To do so, a pulsed laser beam is focused on the specimen using an objective. The high energy of the light ablates the tissue in focus. Many pulses are arrayed in sequence to obtain the desired section cut. The laser hits the specimen for very short intervals of less than one second. This completely eliminates any

heat transfer outside the focus area. A powerful diode laser enables fast, highly precise cutting, even of individual cells. With a 63x magnification objective, a section width of approximately 1µm is attained; with a 150x dry objective, the width is even less than 1µm. The area that is cut out falls into a collection container along with the carrier membrane. In the Leica Microsystems microdissection system, the precise cut guidance is controlled by prisms and increases in direct proportion to the objective magnification.

An important consideration when combining laser microdissection and qPCR is maintaining the integrity of the mRNA from the time the specimen is obtained until the time of the qPCR. Deller and colleagues Drs. Guido J. Burbach, Charlotte Nolte-Uhl, and Stefanie Frank developed a rapid immunofluorescence protocol that permits minimal staining and incubation periods and rapid further processing before dissection, while at the same time ensuring high mRNA quality [3]. To obtain sufficient mRNA for analysis, 60 astrocytes were dissected. After isolation and transcription into cDNA, the specimens were analyzed using qPCR.

Astrocytes respond to lesions in a differentiated manner

The results show clear differences in the height of the measured gene expression between dissected individual cells and dissected tissue areas. While an 18-fold increase in the GFAP mRNA over the control specimens was measured in the astro-

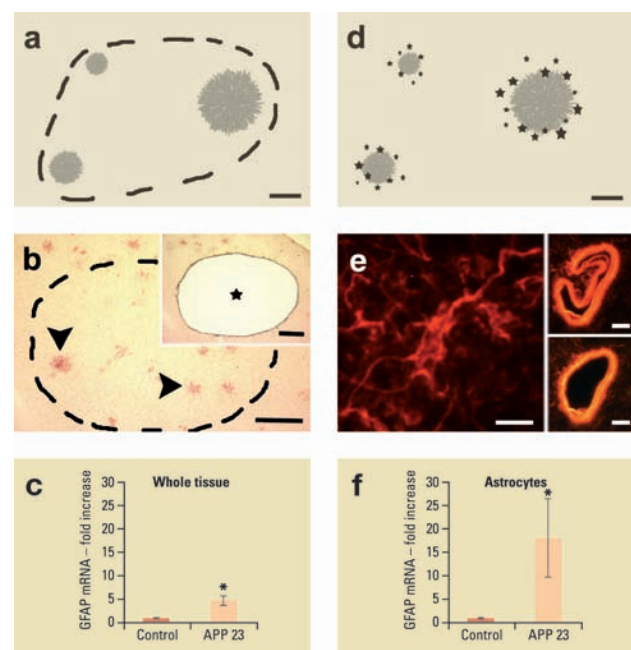
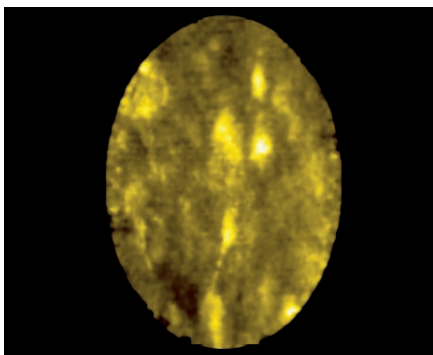
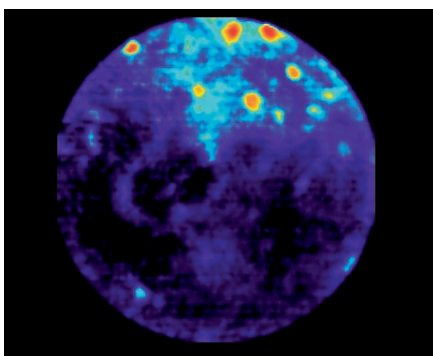
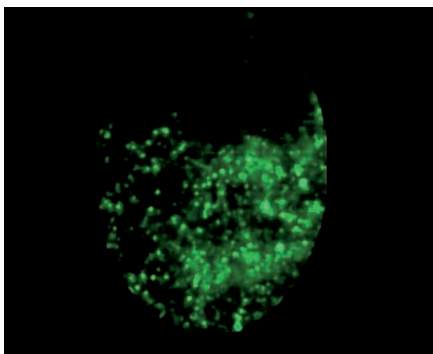


Fig. 2: Increased expression of GFAP mRNA in the vicinity of amyloid plaques. **a, b:** Plaques in the cortex of APP23 mice (**a**: schematic, **b**: cortex tissue stained with Congo Red, before and after dissection). **c:** GFAP mRNA in tissue containing plaque is 5 times greater than that in control specimens. **d, e:** GFAP-marked astrocytes in immediate vicinity of plaque (**d**: schematic, **e**: fluorescence photograph before, during (top right) and after dissection (bottom right, with autofluorescent residual tissue)). **f:** GFAP mRNA in astrocytes is 18 times greater than that in control specimens. $P < 0.05$; size of scale bar = 50µm in **a, d**; 200 or 250µm in **b**; 10 or 5µm in **e**.

Technology Fast Track

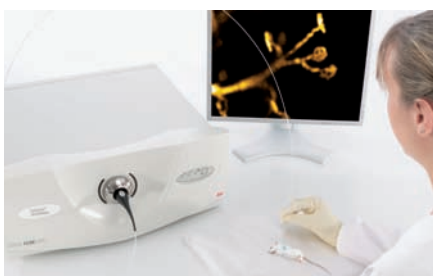
Deep Brain Imaging

Watch Life as it Happens with the Leica FCM1000 Endoscopic Fluorescence Microscope



From top to bottom:
GFP stem cells injected and imaged in living mouse brain, FOV: 300 x 400µm.; Neurons loaded with Oregon Green BAPTA 1 for calcium imaging, FOV: 300µm.; Neurons of transgenic mouse expressing Clomeleo, FOV: 300 x 400µm.

Images courtesy of T. Kuner, G. Feng, G. Augustine, MPI, Heidelberg, Germany; P. Vincent, R. Lambert, L. Bourgeois, ERT 1059, Pierre et Marie Curie University, Paris, France; Dr. Cheng, Taichung Veteran's General Hospital, Taichung, Taiwan ROC; Prof. Haydon, School of Medicine, University of Pennsylvania.



The Leica FCM1000

As biomedical research progresses, the emphasis on *in vivo* imaging and analysis is growing. Today, observing life as it happens is the dream of most researchers. From whole body imaging to intra-vital microscopy, many solutions help to approach this goal. However, there are always trade-offs between high resolution and low invasiveness. Leica Microsystems answers this challenge with the Leica FCM1000, the very first imaging solution developed for and fully adapted to *in vivo* and *in situ* small animal imaging.

Real-time imaging anywhere in the living animal

In vivo observation of live processes requires a high degree of miniaturization for minimally invasive access as well as an ultra-high frame rate for real-time dynamic recording. Using fibered microprobes, the Leica FCM1000 is the only solution for imaging cells anywhere in the brain of living animals with minimal disturbance. By combining minimally invasive access to any structure of interest and an exceptionally high frame rate, the instrument enables real-time recording of live processes as they happen, where they happen. A simple contact with the tissue of interest is enough to generate high-speed documentation of cellular or vascular events.

Intuitive and easy to use

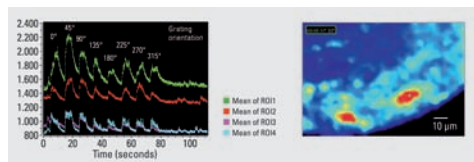
Specifically developed for *in situ* observations of cellular or vascular events, the Leica FCM1000 provides a highly intuitive user interface. From instrument setup to image acquisition and data analysis, the system is easy to use and complements current imaging practices with true *in vivo* imaging.

A unique tool for in situ brain imaging

With fibered microprobes that feature diameters as small 0.3mm, the Leica FCM1000 enables *in situ* imaging at any location in the brain of lab animals. Leica FM Microprobes are readily compatible with a stereotaxic approach and can be attached to most micropipette holders. The shaped tip allows minimal disturbance to the tissue and good tissue-to-microprobe contact. The Leica FCM1000 is a complete solution that combines high performance with very limited invasiveness and a user-friendly environment. The system can be combined and synchronized to fMRI, EEG, electrode recording, and other imaging devices. Applications in brain research cover a wide range of observations of cells or micro-vessels in the brain or olfactory bulb, including:

- Neuronal activity
- Reporter gene expression
- Migration and fate of stem cells and neuron precursors
- Functional changes in micro-vascularization
- Blood/brain barrier studies

Integrated image and data extraction tools offer qualitative and quantitative analysis of experiments.



Neuron activity recording with calcium sensitive dyes: neuron activation in a cat's visual cortex with fibered fluorescence microscopy. Neuron orientation specificity was checked by presenting to the anesthetized animal stripes with changing orientations, in cycles of five seconds. Courtesy: Aaron Kerlin, Kenichi Ohki, Clay Reid, Harvard Medical School, Boston, MA.

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Modulation Contrast

by Lon Nelson, Marketing Manager, Leica Microsystems

Question: What is Modulation Contrast and when do I use it?

Answer: Contrast makes life visible. In the past, scientists struggled to visualize cells, bacteria, and protozoa because their microscopes lacked the ability to impart contrast on samples that were otherwise transparent.

Think of light as sinusoidal waves. The human eye can detect differences in wavelength (color) and differences in amplitude (intensity or bright vs. dark) but not changes in phase, e.g., the point of light wave cresting with the same characteristics. This is why when white light shines through a microscope, which has traversed through an unstained cell, it will not provide any visible detail that would allow you to discern the cell from the white background.

Today's advanced microscopes can provide the contrast needed to view unstained specimens when used with modern technologies. One of these technologies is called Modulation Contrast, often referred to as Hoffman Modulation Contrast. Modulation contrast effectively takes the changes in phase that light experiences when passing through an unstained cell and displays that phase change as a difference in intensity that you can see.

Modulation contrast requires two critical components: a slit illumination diaphragm placed before the specimen and a modulator placed after the specimen (Figure 1).

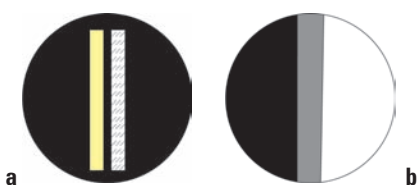


Figure 1: **a:** The slit illumination disk placed before the specimen to provide oblique lighting; **b:** The variable position (y-direction) modular placed after the specimen to adjust the contrast effect.

The slit illumination diaphragm coupled with the microscope's condenser provides oblique (off-axis) illumination to the sample. When this light encounters the unstained specimen, it is diffracted at different degrees based upon the varying thickness and composition of the specimen; then, the light is captured by the microscope's objective lens. The modulator accentuates these differences in diffraction and 'blocks' some of the unhindered light that did not encounter the specimen, which creates contrast through intensity



Figure 2: Unstained *c.elegans* nematode in modulation contrast

variations. See Figure 2 for a general look at how modulation contrast turns phase gradients into brightness gradients. Although an ingenious technique, modulation contrast was difficult to use in the past because the modulator was actually mounted inside of the objective lens. This design

severely limited a lens' use with brightfield and fluorescence imaging. Leica Microsystems has introduced Integrated Modulation Contrast (IMC), which resolves this issue by moving the modulator out of the lens and allows the user to generate high-quality images with any contrast method using the same objective.

When should I use Modulation Contrast?

Use modulation contrast for imaging unstained specimens in plastic containers and when you require higher resolution or better edge detection than phase contrast offers. And, although it is substantially more expensive and will not work with plastic, Differential Interference Contrast (DIC) offers the best resolution of all the techniques.

A practical example for using modulation contrast is imaging a mouse embryo's pronucleus during a microinjection technique. Phase contrast does not offer the resolution to image this sub-cellular structure and DIC cannot be employed as most microinjection techniques use plastic specimen containers.

For a quick review of white light contrasting techniques, see Table 1.

Table 1. Comparison of Different White Light Contrast Techniques

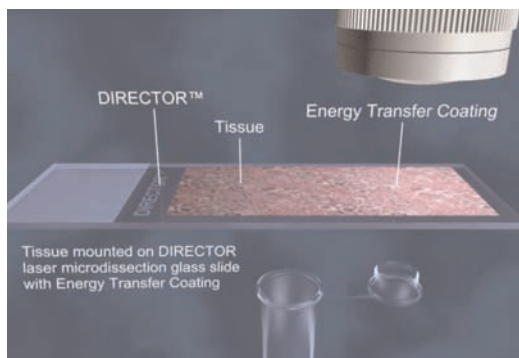
Contrast Technique	Typical Cost	Works with Plastic?	Details
Phase	Least	Yes	Lowest resolution 'Halo' obscures edges Most used technique
Modulation	More	Yes	Higher resolution Better edge detection Least used technique
Differential Interference	Most	No (glass only)	Highest resolution Edges very crisp Popular, but expensive



The Private Eye

In this section of our newsletter, we highlight interesting, emerging technology or technology secrets. This time we focus on new slides for use in laser microdissection.

The DIRECTOR™ slides, from Expression Pathology, are faster, more precise and accurate than any other microdissection technology. They dramatically simplify collection of tissue features from all kinds of tissue, and are well suited for both genomic and proteomic applications. To find out more about this technology and its use with the Leica LMD6000 Laser MicroDissection tool, please click on this link...<http://expressionpathology.com/downloads.shtml>



Courtesy of: Peter Tunon, Vice President Sales and Marketing
Expression Pathology Inc., Gaithersburg, MD
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Laser Microdissection

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cytes cut out of the immediate plaque environment, the GFP mRNA in the dissected tissue increased only 5-fold (Fig. 2). Deller's workgroup was able for the first time to describe and quantify GFP mRNA expression in isolated astrocytes for acute and chronic brain injuries and in Alzheimer's mice. The researchers were able to infer from the results that the type and severity of the damage is an important factor for the strength of the postlesional astrocyte reaction in the brain [2].

Unwanted axonal sprouting due to neurotrophic factors

The work group used a comparable combination of methods when analyzing axonal sprouting in Alzheimer's mice [4, 5]. The BDNF (brain-derived neurotrophic factor) can trigger axonal growth and also changes in Alzheimer's disease. Plaque tissues were dissected to study the gene expression of BDNF mRNA in Alzheimer's mice. In addition, a 30µm wide ring of tissue surrounding the plaques was cut out. Dissected specimens from plaque-free tissue areas served as the control (Fig. 1, a-d). The quantitative PCR analysis identified a gradient of BDNF mRNA (Fig. 1, e, f) in the direction of the plaques. These results, along with the immunohistochemical findings, indicate that in the immediate vicinity of amyloid plaques, glial cells are activated that synthesize and release BDNF. BDNF – probably along with other factors – could have a stimulating effect on axonal growth. Axons could grow along the BDNF gradient to the plaque. The inflammatory and toxic environment of the plaques could damage the growing axons and cause disturbances in synaptic connections.

High sensitivity of the methodology confirmed

The experiments are an impressive demonstration of the advantages of laser microdissection when used for cell-specific and molecule-specific analyses. Deller and his colleagues praise the precision and ease of use of the Leica Microsystems laser microdissection system as well as its wide variety of applications – and foresee even more interesting possibilities in the future. If the technology can be further refined and new upstream and downstream methods that are better matched to the systems become available, laser microdissection could also become established in proteomics research.

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