

CHAPTER 3 INTRODUCTION TO LASER SCANNING MICROSCOPY

CONTENTS

	Page
3 INTRODUCTION TO Laser Scanning Microscopy.....	3-3
3.1 Principle of Laser Scanning Microscopy.....	3-3
3.2 Three-Dimensional Presentations of LSM Image Stacks	3-4
3.3 Optical Diagram of the LSM 510 (Schematic).....	3-6
3.4 Performance Features of the LSM 510.....	3-7
3.4.1 Optical and Mechanical Aspects	3-7
3.4.2 Microscope Equipment of the LSM 510 System	3-8
3.4.3 Computer Hardware and Software	3-10

3 INTRODUCTION TO LASER SCANNING MICROSCOPY

3.1 Principle of Laser Scanning Microscopy

To yield information on their inner structure by conventional transmitted-light microscopy, specimens have to be very thin and translucent; otherwise image definition will be poor. In many cases it is a problem to satisfy these requirements.

The essential considerations have led to trailblazing changes in conventional microscopy and supplied a successful solution to the above problem.

- Unlike the practice of even illumination in conventional microscopy, the LSM technique projects the light of a point light source (a laser) through a high-NA objective onto a certain object plane of interest as a nearly diffraction-limited focus. However, if not for another "trick", the stray light produced outside the object plane, or the fluorescence of fluorescent specimens, would disturb the in-focus image of object point of interest, resulting in a blurred image of poor contrast. The problem therefore is how to capture only the light coming immediately from the object point in focus, while obstructing the light coming from out-of-focus areas of the specimen.
- The light reflected, or the fluorescence light produced, at the focus of the high-NA objective is projected onto a variable pinhole diaphragm by the same objective and a tube lens. The focus inside the specimen and the pinhole are situated at optically conjugate points (**confocal imaging**). The decisive advantage of this arrangement is the fact that essentially no other light than that coming from the object plane of interest can pass the narrow pinhole and be registered by a detector. Unwanted light coming from other specimen areas is focused outside the pinhole, which passes only a small fraction of it. The smaller the pinhole, the less stray light or fluorescence from out-of-focus areas will get on the detector. The image point thus generated is largely free from blur caused by unwanted light.

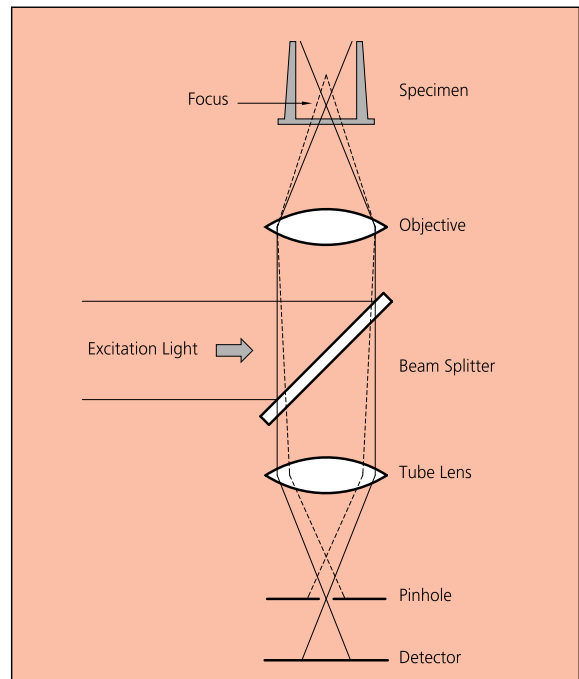


Fig 3-1 Principle of confocal imaging

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- In order to obtain an image of the selected object plane as a whole, it is necessary to scan the object plane in a point-by-point, line-by-line raster by means of an XY light deflection system. The detectors - as a rule, photomultipliers - convert the optical information into electric signals. This allows the image of any object plane to be generated and stored within less than a second. By a defined focusing (Z axis) movement it is possible to look at any object plane of interest. By scanning a succession of object planes in a specimen, a stack of slice images can be produced.

This way, the LSM technique in conjunction with ICS optics (Infinity Color-Corrected System) has brought decisive improvements over conventional microscopy in terms of resolving power and confocal depth contrast:

Object features in the order of 0.2 μm can be resolved, and height differences of less than 0.1 μm made visible, without the use of interference methods.

3.2 Three-Dimensional Presentations of LSM Image Stacks

One of the advantages of the LSM technique is that it can present structures in three dimensions. This opens up many ways to process images. Outlined below are some of the possible methods to extract spatial information from stacks of slice images.

- **Gallery**

The simplest presentation of 3D information is a gallery showing the individual slice images (sections) of a stack arranged side by side, with each slice apart from the next by a defined, selectable interval on the Z axis.

- **Virtually infinite depth of focus**

The entire set of data can be imaged as a single projection. The computer establishes an image composed of all in-focus optical sections. The image produced by this so-called composite method has a virtually infinite depth of focus, since the result is made up of information from in-focus planes only.

- **Rotary animation**

A sequence of projections is computed, with the specimen being apparently rotated by a certain angle from image to image, for example by a full turn about an axis. If such a sequence is displayed on the monitor screen in rapid succession, the visual effect is that of a rotating three-dimensional object.

- **Stereo image pairs**

The computer establishes a pair of images corresponding to those we see with the right and the left eye, respectively. The two images forming the stereo pair can be shown on the monitor side by side. They can be seen as a 3D image with suitable optical aids. Another possibility is to present both images in registration, with one image in the red channel and the other in the green one (anaglyph). Viewed through red and green color filters in a spectacle frame, which only pass the image intended for the respective eye, the two images form a 3D image in the brain

- **Color-coded height slices**
Each level, i.e. each slice is assigned a different color. For direct evaluation, a color scale is shown, indicating the actual height above the bottom slice.
- **Orthogonal sections**
This computation produces a triplet of mutually perpendicular sectional images.
- **Oblique sections**
A section through the stack is made along an oblique plane defined by the selection of five coordinates, i.e. X, Y, Z, angle of rotation, and angle of tilt.
- **Topography** (option)
A computing program for surface topography presentations (as required in materials research) is available.
- **Physiology** (option)
With a special software, kinetic processes can be tracked, which is especially of interest to physiology.
- **Image VisArt** (option)
Three-dimensional display of floating transparent structures (cells) or opaque structures (material)
- **3D Deconvolution** (option)

3.3 Optical Diagram of the LSM 510 (Schematic)

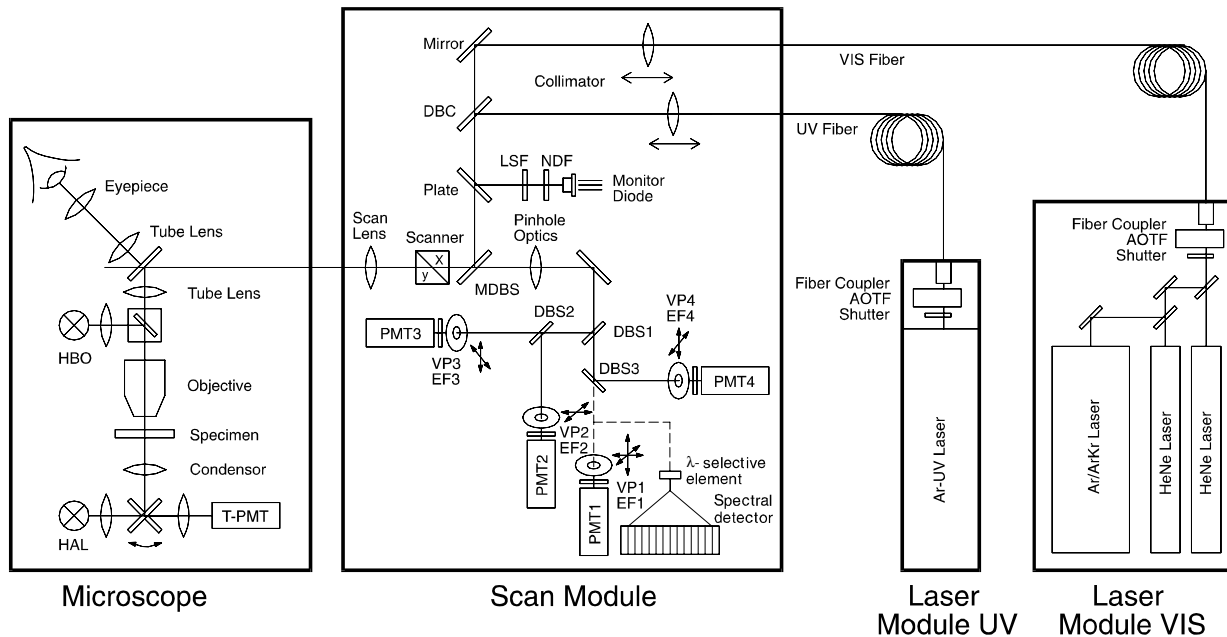


Fig. 3-2 Optical path, schematic (4-channel configuration)

- AOTF Acousto Optical Tunable Filter
- DBC Dichroic Beam Combiner
- DBS Dichroic Beam Splitter
- EF Emission Filter
- HAL Halogen Lamp
- HBO Mercury Vapor Short-Arc Lamp
- LSF Line Selection Filter
- MDBS Main Dichroic Beam Splitter
- NDF Neutral Density Filter
- VP Variable Pinhole
- PMT Photomultiplier
- T-PMT Transmission-Photomultiplier

The diagram above is a schematic representation of the LSM system.

Laser light is focused onto the specimen through an objective in a diffraction-limited mode. Light emitted at the focal plane and at planes below and above it is directed via an XY scanner onto a main dichroic beam splitter (MDBS), which separates the emissions from the excitation light. The fluorescences are separated from each other by a series of dichroic beam splitters (DBS1 ... maximally DBS4) and directed to individual photomultipliers (PMT1 ... maximally PMT4).

3.4 Performance Features of the LSM 510

3.4.1 Optical and Mechanical Aspects

The highly integrated system design makes for the shortest possible optical paths, top-grade optical precision and high stability. The compact scanning module can be fitted to an inverted (Axiovert 200 M BP or SP) or upright (Axioplan 2 imaging MOT) microscope in less than three minutes. On the Axiovert, the scanning module may be mounted either to the base port directly below the microscope or to the side port.

The spectral range available extends from the UV to the IR region.

For the VIS (visible-light) Laser Module, the user can select from up to five lasers with wavelengths of 633, 568, 543, 514, 488, 477, 458 and 405 nm. The UV Laser Module provides wavelengths of 351 and 364 nm. Coupling of the laser light is through polarization-preserving single-mode optical fibers. One variable beam collimator each for the UV and visible ranges provides optimum adaptation of the respective laser wavelength to the objective used and, thus, optimum correction for Z aberrations.

Acousto-optical tunable filters (AOTF) adjust the necessary brightness for up to 6 desired laser lines within microseconds.

A monitor diode permanently registers the laser output; it can be used for the on-line checking of the intensity of the exciting light. This check is also possible selectively for the different wavelengths if a line selection filter is inserted.

The four simultaneous image acquisition channels, usable for reflection or fluorescence, and an additional transmitted-light channel are ideal for the investigation of multiple fluorescence specimens. Separately in each of the four channels, the diameters of the pinholes and their XY positions can be optimized, and the desired emission filter placed into the beam path, by servo-motor control. In the case of pinhole VP1, this adjustment also includes positioning along Z. In the simultaneous registration of multiple fluorescences, identical optical sections can be obtained in each confocal channel. This is of importance, e.g., with the FISH method (fluorescence in-situ hybridization) used for genome analysis in cytogenetic studies.

The microscope's transmitted-light channel is equipped with a photomultiplier, too. It is therefore possible to superimpose a multiple fluorescence image on a brightfield, differential interference or phase image.

A fiber-optic cable connection to external special detectors, such as cooled PMTs or spectrometers, is under development.

In addition to the emission filters for all standard and special applications, available in motor-controlled filter wheels, the user can easily install his own emission filters in two of the channels.

The high-NA C-APOCHROMAT objectives specially developed for the LSM technique reach the physical limit in resolving power, and can be used throughout the 350...700 nm spectral range with the same high quality, producing brilliant images.

A two-mirror scanner system, controlled by a digital signal processor (DSP), offers several advantages. The large deflection angle of the scanning mirrors allows a wide area to be scanned. With a 1.25x objective, the object area scanned is $10 \times 10 \text{ mm}^2$.

The scanning field size can be freely selected between 4×1 and 2048×2048 pixels.

It is possible to rotate the XY scanning field through 360° and carry out XY scans without having to rotate the specimen itself under laser radiation load.

Selection of the specimen detail of interest for zooming is fast and convenient, and the zoomed image is automatically centered. This saves the job of specimen centration with the microscope stage.

Using a bi-directional scanning facility will double the scanning rate to approx. 5 frames/sec (at 512 × 512 pixels); if two different laser wavelengths are used for the two scanning directions (wavelength 1 for left-to-right, and wavelength 2 for right-to-left scanning), two fluorochrome dyes can be viewed and documented in a quasi-simultaneous mode. This will absolutely prevent "bleeding".

3.4.2 Microscope Equipment of the LSM 510 System

The LSM 510 system is equipped either with the inverted Axiovert 200 M BP or SP microscope or with the upright Axioplan 2 imaging MOT, Axiotron 2 or Axioskop 2 FS mot microscopes.

Only the differences from the delivered operating manual "Axiovert 200 M" will be explained here.

(1) Stand

a) The motorized objective nosepiece 5× H DIC is firmly fixed to the stand, where no operating elements can be found for the nosepiece. Operation will be performed via LSM 5 software control. The "Restriction of the nosepiece height to protect the objectives during motorized objective change" is inactivated. The nosepiece will be moved down automatically before each motorized objective change.

b) The reflector mount is motorized and provided with the Axiovert 200 reflector turret. The reflector turret has 5 positions: One transmitting light position, which is identical to the LSM position, and four further positions for fluorescence filter sets (reflector modules). If you want to use more than five conventional fluorescence filter sets, it is advisable to use a further reflector turret. When changing the reflector turret position you must make sure that the turret will click into position, since otherwise the image area will be cut.

c) The stand has a motorized focusing drive (fine coarse). Sensitivity of the focusing drive is adjusted to the delivered objectives by the manufacturer. If you want to use other objectives, sensitivity and parfocality can be adjusted via the Axioset program.

d) The stand features an integrated power supply for the internal motors and stand electronics. The power supply can be switched on at the right side of the stand. External power supply units will be used for the mercury vapor short arc lamp.

e) The analyzer slider for conventional DIC methods will be operated from the right side and is located just below the nosepiece.

When the rod is pushed in, the analyzer is located in the beam path. In the LSM-mode, the analyzer must **not** be located in the beam path, and the analyzer rod must be pulled out.

f) The stands dispose of five additional ports: two side ports, front ports and base ports.

The side port or the front is equipped with the LSM 5 special interface, one of the others with the TV interface. The LSM 510 scanning module can be mounted to the special interface port. Different camera systems can be adapted to the TV interface using the TV adapters 452982/83/92/94/95/97/98-0000-000.

(2) Specimen stages and fine focus drives

a) Mechanical stage

The stage with coaxial drive must be mounted on the right side of the stand.

b) Scanning stage

c) HRZ 200 fine focusing stage

d) Piezo objective focus drive

(3) Transmitted-light illumination

a) The illuminator support contains a security circuit which activates a shutter preventing laser light from reaching the stand when the support is moved to the back. A complementary shutter built in the stand prevents laser light from reaching the eyepieces during the scanning mode.

b) The illuminator support is equipped with a rotary polarizer. The Axiovert description contains the adjustment for the DIC mode during conventional observation.

For scanning in the transmitted-light DIC mode, the polarizer in the transmitted light support works like an analyzer and must be adjusted in such a manner that direct laser light will be blocked.

The conventional analyzer slider in the stand must not be located in the beam path because the laser light is already polarized.

c) A fully motorized, LSM 5 software-controlled switching mirror is mounted as an option on the illuminator support. Alternatively, the light is directed to the LSM 5 T-light detector or enables conventional transmitted-light observation.

d) The focusing screen for conventional transmitted-light is located in a support in front of the halogen lamp housing.

e) Further information on the halogen lamp and the condensers is provided in the Axiovert operating manual.

(4) Reflected light fluorescence

With the exception of the reflector slider, all the Axiovert fluorescence accessories can be used. Further information is provided in the Axiovert operation manual.

(5) Imaging optics

Optovar sliders cannot be used.

The analyzer for the conventional DIC mode will be operated from the right side and is located just below the nosepiece.

Use of sliders with auxiliary objects (473704/14-0000-000) is not possible.

(6) Photo equipment

The stand does not feature an integrated SLR-port, but microscope cameras as described in the Axiovert operation manual can be used.

(7) TV adaptation

The TV port at the side and the tubes can be used as described in the Axiovert operation manual.

The TV interface side port or base port can be used with TV adapters 44 or LSM adapters.

3.4.3 Computer Hardware and Software

The LSM 510 is controlled via a standard high-end Pentium PC. Linking to the electronic control system is made via an ultrafast SCSI interface. The PC comes with the 32-bit WINDOWS NT 4.0 or WINDOWS 2000 operating system.

The instrument is fully motorized, permitting fast change-over between methods as well as automatic operation. Parameters once set or complex examination sequences once established can be saved and reproduced; therefore, complete application programs can be loaded and performed by pushbutton control.

The software of the LSM 510 has two levels. On the simple operator interface level, a result will be achieved after a few prompts; graphical prompting of the user in conjunction with automatic setting of many parameters is an ideal tool for daily routine jobs. The expert level offers perfect facilities for individual settings of functions and parameters.

Conversion of the light signals into a digital image is effected by means of four 12-bit A/D converters, each of which can generate 4096 brightness levels.

The software provides an enormously wide range of image processing functions, including all standard 2D/3D (stereo, projection) functions identical to sophisticated 3D reconstruction capabilities (surface and alpha rendering), digital processing of voxels and 3D measurement functions (surface areas, volumes).

As all files and images are recorded in MS Access databases, elegant image database editing is just as easy as transferring the records to other programs.