Confocal Laser Scanning Microscopy

Optical Image Formation
Electronic Signal Processing
Highlights of Laser Scanning Microscopy

1982
The first Laser Scanning Microscope from Carl Zeiss. The prototype of the LSM 44 series is now on display in the Deutsches Museum in Munich.

1988
The LSM 10 – a confocal system with two fluorescence channels.

1991
The LSM 310 combines confocal laser scanning microscopy with state-of-the-art computer technology.

1992
The LSM 410 is the first inverted microscope of the LSM family.

1997
The LSM 510 – the first system of the LSM 5 family and a major breakthrough in confocal imaging and analysis.

1998
The LSM 510 NLO is ready for multiphoton microscopy.

1999
The LSM 5 PASCAL – the personal confocal microscope.

2000
The LSM is combined with the ConfoCor 2 Fluorescence Correlation Spectroscope.

2001
The LSM 510 META – featuring multispectral analysis.
Confocal Laser Scanning Microscopy

In recent years, the confocal Laser Scanning Microscope (LSM) has become widely established as a research instrument.

The present brochure aims at giving a scientifically sound survey of the special nature of image formation in a confocal LSM.

LSM applications in biology and medicine predominantly employ fluorescence, but it is also possible to use the transmission mode with conventional contrasting methods, such as differential interference contrast (DIC), as well as to overlay the transmission and confocal fluorescence images of the same specimen area.

Another important field of application is materials science, where the LSM is used mostly in the reflection mode and with such methods as polarization.

Confocal microscopes are even used in routine quality inspection in industry. Here, confocal images provide an efficient way to detect defects in semiconductor circuits.
Following a description of the fundamental differences between a conventional and a confocal microscope, this monograph will set out the special features of the confocal LSM and the capabilities resulting from them.

The conditions in fluorescence applications will be given priority treatment throughout.

**Image generation**

The complete generation of the two-dimensional object information from the focal plane (object plane) of a confocal LSM essentially comprises three process steps:

1. Line-by-line scanning of the specimen with a focused laser beam deflected in the X and Y directions by means of two galvanometric scanners.
2. Pixel-by-pixel detection of the fluorescence emitted by the scanned specimen details, by means of a photomultiplier tube (PMT).
3. Digitization of the object information contained in the electrical signal provided by the PMT (for presentation, the image data are displayed, pixel by pixel, from a digital matrix memory to a monitor screen).

![Diagram](image.png)
Introduction

Scanning process

In a conventional light microscope, object-to-image transformation takes place simultaneously and parallel for all object points. By contrast, the specimen in a confocal LSM is irradiated in a point-wise fashion, i.e. serially, and the physical interaction between the laser light and the specimen detail irradiated (e.g. fluorescence) is measured point by point. To obtain information about the entire specimen, it is necessary to guide the laser beam across the specimen, or to move the specimen relative to the laser beam, a process known as scanning. Accordingly, confocal systems are also known as point-probing scanners.

To obtain images of microscopic resolution from a confocal LSM, a computer and dedicated software are indispensable.

The descriptions below exclusively cover the point scanner principle as implemented, for example, in Carl Zeiss laser scanning microscopes. Configurations in which several object points are irradiated simultaneously are not considered.

Confocal beam path

The decisive design feature of a confocal LSM compared with a conventional microscope is the confocal aperture (usually called pinhole), which is arranged in a plane conjugate to the intermediate image plane and, thus, to the object plane of the microscope. As a result, the detector (PMT) can only detect light that has passed the pinhole. The pinhole diameter is variable; ideally, it is infinitely small, and thus the detector looks at a point (point detection).

As the laser beam is focused to a diffraction-limited spot, which illuminates only a point of the object at a time, the point illuminated and the point observed (i.e. image and object points) are situated in conjugate planes, i.e. they are focused onto each other. The result is what is called a confocal beam path (see figure 2).

Pinhole

Depending on the diameter of the pinhole, light coming from object points outside the focal plane is more or less obstructed and thus excluded from detection. As the corresponding object areas are invisible in the image, the confocal microscope can be understood as an inherently depth-discriminating optical system.

By varying the pinhole diameter, the degree of confocality can be adapted to practical requirements. With the aperture fully open, the image is nonconfocal. As an added advantage, the pinhole suppresses stray light, which improves image contrast.

Fig. 2  Beam path in a confocal LSM. A microscope objective is used to focus a laser beam onto the specimen, where it excites fluorescence, for example. The fluorescent radiation is collected by the objective and efficiently directed onto the detector via a dichroic beamsplitter. The interesting wavelength range of the fluorescence spectrum is selected by an emission filter, which also acts as a barrier blocking the excitation laser line. The pinhole is arranged in front of the detector, on a plane conjugate to the focal plane of the objective. Light coming from planes above or below the focal plane is out of focus when it hits the pinhole, so most of it cannot pass the pinhole and therefore does not contribute to forming the image.
Optical slices

With a confocal LSM it is therefore possible to exclusively image a thin optical slice out of a thick specimen (typically, up to 100 µm), a method known as optical sectioning. Under suitable conditions, the thickness (Z dimension) of such a slice may be less than 500 nm. The fundamental advantage of the confocal LSM over a conventional microscope is obvious: in conventional fluorescence microscopy, the image of a thick biological specimen will only be in focus if its Z dimension is not greater than the wave-optical depth of focus specified for the respective objective. Unless this condition is satisfied, the in-focus image information from the object plane of interest is mixed with out-of-focus image information from planes outside the focal plane. This reduces image contrast and increases the share of stray light detected. If multiple fluorescences are observed, there will in addition be a color mix of the image information obtained from the channels involved (figure 3, left).

A confocal LSM can therefore be used to advantage especially where thick specimens (such as biological cells in tissue) have to be examined by fluorescence. The possibility of optical sectioning eliminates the drawbacks attached to the observation of such specimens by conventional fluorescence microscopy. With multicolor fluorescence, the various channels are satisfactorily separated and can be recorded simultaneously.

With regard to reflective specimens, the main application is the investigation of the topography of 3D surface textures. Figure 3 demonstrates the capability of a confocal Laser Scanning Microscope.

Fig. 3. Non-confocal (left) and confocal (right) image of a triple-labeled cell aggregate (mouse intestine section). In the non-confocal image, specimen planes outside the focal plane degrade the information of interest from the focal plane, and differently stained specimen details appear in mixed color. In the confocal image (right), specimen details blurred in non-confocal imaging become distinctly visible, and the image throughout is greatly improved in contrast.
3rd dimension

In addition to the possibility to observe a single plane (or slice) of a thick specimen in good contrast, optical sectioning allows a great number of slices to be cut and recorded at different planes of the specimen, with the specimen being moved along the optical axis (Z) by controlled increments. The result is a 3D data set, which provides information about the spatial structure of the object. The quality and accuracy of this information depend on the thickness of the slice and on the spacing between successive slices (optimum scanning rate in Z direction = 0.5x the slice thickness). By computation, various aspects of the object can be generated from the 3D data set (3D reconstruction, sections of any spatial orientation, stereo pairs etc.). Figure 4 shows a 3D reconstruction computed from a 3D data set.

Time series

A field of growing importance is the investigation of living specimens that show dynamic changes even in the range of microseconds. Here, the acquisition of time-resolved confocal image series (known as time series) provides a possibility of visualizing and quantifying the changes. The following section (Part 1, page 6 ff) deals with the purely optical conditions in a confocal LSM and the influence of the pinhole on image formation. From this, ideal values for resolution and optical slice thickness are derived.

Part 2, page 16 ff limits the idealized view, looking at the digitizing process and the noise introduced by the light as well as by the optoelectronic components of the system.

The table on page 15 provides a summary of the essential results of Part 1. A schematic overview of the entire content and its practical relevance is given on the poster inside this brochure.

Fig. 4  3D projection reconstructed from 108 optical slices of a three-dimensional data set of epithelium cells of a lacrimal gland. Actin filaments of myoepithelial cells marked with BODIPY-FL phallacidin (green), cytoplasm and nuclei of acinar cells with ethidium homodimer-1 (red).

Fig. 5  Gallery of a time series experiment with Kaede-transfected cells. By repeated activation of the Kaede marker (green-to-red color change) in a small cell region, the entire green fluorescence is converted step by step into the red fluorescence.
Point Spread Function

In order to understand the optical performance characteristics of a confocal LSM in detail, it is necessary to have a closer look at the fundamental optical phenomena resulting from the geometry of the confocal beam path. As mentioned before, what is most essential about a confocal LSM is that both illumination and observation (detection) are limited to a point.

Not even an optical system of diffraction-limited design can image a truly point-like object as a point. The image of an ideal point object will always be somewhat blurred, or “spread” corresponding to the imaging properties of the optical system. The image of a point can be described in quantitative terms by the point spread function (PSF), which maps the intensity distribution in the image space.

Where the three-dimensional imaging properties of a confocal LSM are concerned, it is necessary to consider the 3D image or the 3D-PSF.

In the ideal, diffraction-limited case (no optical aberrations, homogeneous illumination of the pupil – see Details “Pupil Illumination”), the 3D-PSF is of comet-like, rotationally symmetrical shape.

For illustration, Figure 6 shows two-dimensional sections (XZ and XY ) through an ideal 3D-PSF. From the illustration it is evident that the central maximum of the 3D-PSF, in which 86.5% of the total energy available in the pupil are concentrated, can be described as an ellipsoid of rotation. For considerations of resolution and optical slice thickness it is useful to define the half-maximum area of the ellipsoid of rotation, i.e. the well-defined area in which the intensity of the 3D point image in axial and lateral directions has dropped to half of the central maximum.
Any reference to the PSF in the following discussion exclusively refers to the half-maximum area. Quantitatively the half-maximum area is described in terms of the full width at half maximum (FWHM), a lateral or axial distance corresponding to a 50% drop in intensity.

The total PSF (PSF\text{tot}) of a confocal microscope behind the pinhole is composed of the PSFs of the illuminating beam path (PSF\text{ill}; point illumination) and the detection beam path (PSF\text{det}; point detection). Accordingly, the confocal LSM system as a whole generates two point images: one by projecting a point light source into the object space, the other by projecting a point detail of the object into the image space. Mathematically, this relationship can be described as follows:

\[
\text{PSF}_{\text{tot}}(x,y,z) = \text{PSF}_{\text{ill}}(x,y,z) \cdot \text{PSF}_{\text{det}}(x,y,z)
\]

PSF\text{ill} corresponds to the light distribution of the laser spot that scans the object. Its size is mainly a function of the laser wavelength and the numerical aperture of the microscope objective. It is also influenced by diffraction at the objective pupil (as a function of pupil illumination) and the aberrations of all optical components integrated in the system. (Note: In general, these aberrations are low, having been minimized during system design).

Moreover, PSF\text{ill} may get deformed if the laser focus enters thick and light-scattering specimens, especially if the refractive indices of immersion liquid and mounting medium are not matched and/or if the laser focus is at a great depth below the specimen surface (see Hell, S., et al., [9]).

PSF\text{det} is also influenced by all these factors and, additionally, by the pinhole size. For reasons of beam path efficiency (see Part 2), the pinhole is never truly a point of infinitely small size and thus PSF\text{det} is never smaller in dimension than PSF\text{ill}. It is evident that the imaging properties of a confocal LSM are determined by the interaction between PSF\text{ill} and PSF\text{det}. As a consequence of the interaction process, PSF\text{tot} ≤ PSF\text{ill}.

With the pinhole diameter being variable, the effects obtained with small and big pinhole diameters must be expected to differ. In the following sections, various system states are treated in quantitative terms.

From the explanations made so far, it can also be derived that the optical slice is not a sharply delimited body. It does not start abruptly at a certain Z position, nor does it end abruptly at another. Because of the intensity distribution along the optical axis, there is a continuous transition from object information suppressed and such made visible.

Accordingly, the out-of-focus object information actually suppressed by the pinhole also depends on the correct setting of the image processing parameters (PMT high voltage, contrast setting). Signal overdrive or excessive offset should be avoided.
Resolution and Confocality

Wherever quantitative data on the resolving power and depth discrimination of a confocal LSM are specified, it is necessary to distinguish clearly whether the objects they refer to are point-like or extended, and whether they are reflective or fluorescent. These differences involve distinctly varying imaging properties. Fine structures in real biological specimens are mainly of a filiform or point-like fluorescent type, so that the explanations below are limited to point-like fluorescent objects. The statements made for this case are well applicable to practical assignments.

As already mentioned, the pinhole diameter plays a decisive role in resolution and depth discrimination. With a pinhole diameter greater than 1 AU (AU = Airy unit – see Details “Optical Coordinates”), the depth discriminating properties under consideration are essentially based on the law of geometric optics (geometric-optical confocality). The smaller the pinhole diameter, the more PSFillus approaches the order of magnitude of PSFdet. In the limit case (PH < 0.25 AU), both PSFs are approximately equal in size, and wave-optical image formation laws clearly dominate (wave-optical confocality).

Figure 7 illustrates these concepts. It is a schematic representation of the half-intensity areas of PSFillus and PSFdet at selected pinhole diameters.

Depending on which kind of confocality dominates, the data and computation methods for resolution and depth discrimination differ. A comparison with image formation in conventional microscopes is interesting as well. The following sections deal with this in detail.
Resolution

Resolution, in case of large pinhole diameters (PH > 1 A.U.), is meant to express the separate visibility, both laterally and axially, of points during the scanning process. Imagine an object consisting of individual points: all points spaced closer than the extension of PSF, are blurred (spread), i.e. they are not resolved.

Quantitatively, resolution results from the axial and lateral extension of the scanning laser spot, or the elliptical half-intensity area of PSF, On the assumption of homogeneous pupil illumination, the following equations apply:

Axial:

\[
\text{FWHM}_{\text{axial}} = \frac{0.88 \cdot \lambda_{\text{exc}}}{n - n^2 - NA^2} \tag{2}
\]

\[
\approx \frac{1.77 \cdot n \cdot \lambda_{\text{exc}}}{NA^2} \tag{2a}
\]

If NA < 0.5, equation (2) can be approximated by:

Lateral:

\[
\text{FWHM}_{\text{lateral}} = \frac{0.51 \cdot \lambda_{\text{exc}}}{NA} \tag{3}
\]

At first glance, equations (2a) and (3) are not different from those known for conventional imaging (see Beyer, H., [3]): it is striking, however, that the resolving power in the confocal microscope depends only on the wavelength of the illuminating light, rather than exclusively on the emission wavelength as in the conventional case.

Compared to the conventional fluorescence microscope, confocal fluorescence with large pinhole diameters leads to a gain in resolution by the factor \(\lambda_{\text{exc}}/\lambda_{\text{em}}\) via the Stokes shift.
Let the statements made on PSF so far be further illustrated by the figure on the left. It shows a section through the resulting diffraction pattern surrounding the focus on the illumination side (PSF\_illumination). The lines include areas of equal brightness (isophote presentation). The center has a normalized intensity of 1. The real relationships result by rotation of the section about the vertical (Z) axis. Symmetry exists relative to the focal plane as well as to the optical axis. Local intensity maxima and minima are conspicuous. The dashed lines mark the range covered by the aperture angle of the microscope objective used.

For the considerations in this chapter, only the area inside the red line, i.e. the area at half maximum, is of interest.
Geometric optical confocality

Optical slice thickness (depth discrimination) and stray light suppression (contrast improvement) are basic properties of a confocal LSM, even if the pinhole diameter is not an ideal point (i.e., not infinitely small). In this case, both depth discrimination and stray light suppression are determined exclusively by PSF$_\text{det}$. This alone brings an improvement in the separate visibility of object details over the conventional microscope.

Hence, the diameter of the corresponding half-intensity area and thus the optical slice thickness is given by:

$$\text{FWHM}_{\text{det,axial}} = \frac{1}{2} \sqrt{\left( \frac{0.88 \cdot \lambda_{\text{em}}}{\lambda_{\text{em}}^2 (\pi n)^2} \right) + \left( \frac{2 \cdot n \cdot \text{PH}}{\text{NA}} \right)^2}.$$  \hspace{1cm} (4)

Equation (4) shows that the optical slice thickness comprises a geometric-optical and a wave-optical term. The wave-optical term (first term under the root) is of constant value for a given objective and a given emission wavelength. The geometric-optical term (second term under the root) is dominant; for a given objective it is influenced exclusively by the pinhole diameter.

Likewise, in the case of geometric-optical confocality, there is a linear relationship between depth discrimination and pinhole diameter. As the pinhole diameter is constricted, depth discrimination improves (i.e., the optical slice thickness decreases).

A graphical representation of equation (4) is illustrated in Figure 9. The graph shows the geometric-optical term alone (blue line) and the curve resulting from eq. 4 (red line). The difference between the two curves is a consequence of the wave-optical term.

Above a pinhole diameter of 1 AU, the influence of diffraction effects is nearly constant and equation (4) is a good approximation to describe the depth discrimination. The interaction between PSF$_\text{i}$ and PSF$_\text{det}$ becomes manifest only with pinhole diameters smaller than 1 AU.

Let it be emphasized that in case of geometric optical confocality the diameters of the half-intensity area of PSF$_\text{det}$ allow no statement about the separate visibility of object details in axial and lateral direction.

In the region of the optical section (FWHM$_{\text{det,axial}}$), object details are resolved (imaged separately) only unless they are spaced not closer than described by equations (2) / (2a) / (3).
Wave-optical confocality

If the pinhole is closed down to a diameter of <0.25 AU (virtually "infinitely small"), the character of the image changes. Additional diffraction effects at the pinhole have to be taken into account, and PSF\_det (optical slice thickness) shrinks to the order of magnitude of PSF\_ill (Z resolution) (see also figure 7c).

In order to achieve simple formulae for the range of smallest pinhole diameters, it is practical to regard the limit of PH = 0 at first, even though it is of no practical use. In this case, PSF\_det and PSF\_ill are identical.

The total PSF can be written as

$$PSF_{\text{tot}}(x,y,z) = (PSF_{\text{ill}}(x,y,z))^2$$ \hfill (5)

In fluorescence applications it is furthermore necessary to consider both the excitation wavelength $\lambda_{\text{exc}}$ and the emission wavelength $\lambda_{\text{em}}$. This is done by specifying a mean wavelength $\bar{\lambda}$:

$$\bar{\lambda} = \sqrt{\frac{\lambda_{\text{em}} \cdot \lambda_{\text{exc}}}{\lambda_{\text{em}} + \lambda_{\text{exc}}}}$$ \hfill (6)

Thus, equations (2) and (3) for the widths of the axial and lateral half-intensity areas are transformed into:

Axial:

$$\text{FWHM}_{\text{axial}} = \frac{0.64 \cdot \bar{\lambda}}{(n^2 - NA^2)}$$ \hfill (7)

If $NA < 0.5$, equation (7) can be approximated by

$$\approx \frac{1.28 \cdot n}{NA}$$ \hfill (7a)

Lateral:

$$\text{FWHM}_{\text{lateral}} = \frac{0.37 \cdot \bar{\lambda}}{NA}$$ \hfill (8)

Note:

With the object being a mirror, the factor in equation 7 is 0.45 (instead of 0.64), and 0.88 (instead of 1.28) in equation 7a. For a fluorescent plane of finite thickness, a factor of 0.7 can be used in equation 7. This underlines that apart from the factors influencing the optical slice thickness, the type of specimen also affects the measurement result.
From equations (7) and (7a) it is evident that depth resolution varies linearly with the refractive index \( n \) of the immersion liquid and with the square of the inverse value of the numerical aperture of the objective \( (\text{NA} = n \cdot \sin(\alpha)) \).

To achieve high depth discrimination, it is important, above all, to use objectives with the highest possible numerical aperture.

As an \( \text{NA} > 1 \) can only be obtained with an immersion liquid, confocal fluorescence microscopy is usually performed with immersion objectives (see also figure 11).

A comparison of the results stated before shows that axial and lateral resolution in the limit of \( \text{PH} = 0 \) can be improved by a factor of 1.4. Furthermore it should be noted that, because of the wave-optical relationships discussed, the optical performance of a confocal LSM cannot be enhanced infinitely. Equations (7) and (8) supply the minimum possible slice thickness and the best possible resolution, respectively.

From the applications point of view, the case of strictly wave-optical confocality \( (\text{PH} = 0) \) is irrelevant (see also Part 2).

By merely changing the factors in equations (7) and (8) it is possible, though, to transfer the equations derived for \( \text{PH} = 0 \) to the pinhole diameter range up to 1 AU, to a good approximation. The factors applicable to particular pinhole diameters can be taken from figure 10.

It must also be noted that with \( \text{PH} < 1 \text{AU} \), a distinction between optical slice thickness and resolution can no longer be made. The thickness of the optical slice at the same time specifies the resolution properties of the system. That is why in the literature the term of depth resolution is frequently used as a synonym for depth discrimination or optical slice thickness. However, this is only correct for pinhole diameters smaller than 1 AU.

To conclude the observations about resolution and depth discrimination (or depth resolution), the table on page 15 provides an overview of the formulary relationships developed in Part 1. In addition, figure 11a shows the overall curve of optical slice thickness for a microscope objective of \( \text{NA} = 1.3 \) and \( n = 1.52 (\lambda = 496 \text{ nm}) \).

In figure 11b-d, equation (7) is plotted for different objects and varied parameters (\( \text{NA}, \lambda, n \)).
Fig. 11

a) Variation of pinhole diameter

b) Variation of numerical aperture

c) Variation of wavelength ($\lambda$)

d) Variation of refractive index

Optical slice (NA = 1.3; $n = 1.52$; $\lambda = 496$ nm)

Depth resolution (PH = 0; NA = 1.3; $n = 1.52$)

Depth resolution (PH = 0; NA = 0.8; $n = 1.52$)

Depth resolution (PH = 0; NA = 1.3; $n = 1.52$)

Pinhole diameter [µm]

Wavelength [nm]

Refractive index of immersion liquid
### Overview

#### Conventional microscopy

1. **Optical slice thickness not definable**
   
   With a conventional microscope, unlike in confocal microscopy, sharply defined images of "thick" biological specimens can only be obtained if their Z dimension is not greater than the wave-optical depth of field specified for the objective used. Depending on specimen thickness, object information from the focal plane is mixed with blurred information from out-of-focus object zones.

   Optical sectioning is not possible; consequently, no formula for optical slice thickness can be given.

#### Confocal microscopy

1. **Optical slice thickness**
   
   The term results as the FWHM of the total PSF – the pinhole acts according to wave optics.

   \[
   \lambda \sqrt{\frac{1}{n^2 - n^2 A^2}} \left( \frac{n - \text{PH}}{n} \right) \]

   Corresponds to the FWHM of the intensity distribution behind the pinhole (PSFdet). The FWHM results from the emission-side diffraction pattern and the geometric-optical effect of the pinhole. Here, PH is the variable object-side pinhole diameter in µm.

   The factor 0.64 applies only to a fluorescent point object.

2. **Axial resolution**

   FWHM of PSF in Z direction. No influence by the pinhole.

3. **Approximation to 2. for NA < 0.5**

   1.77 \( \frac{n \lambda}{NA^2} \)

   As optical slice thickness and resolution are identical in this case, depth resolution is often used as a synonym.

4. **Lateral resolution**

   FWHM of the diffraction pattern in the intermediate image – referred to the object plane in X/Y direction.

   \[
   \frac{0.57 \lambda}{NA} \]

   The term results as the FWHM of the total PSF -- the pinhole acts according to wave optics. \( \lambda \) stands for a mean wavelength – see the text body above for the exact definition.

   The factor 0.64 applies only to a fluorescent point object.

All data in the table refer to quantities in the object space and apply to a fluorescent point object.

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1) PH <= is meant to express a pinhole diameter of < 4–5 AU.
Sampling and Digitization

After the optical phenomena have been discussed in Part 1, Part 2 takes a closer look at how the digitizing process and system-inherent sources of noise limit the performance of the system. As stated in Part 1, a confocal LSM scans the specimen surface point by point. This means that an image of the total specimen is not formed simultaneously, with all points imaged in parallel (as, for example, in a CCD camera), but consecutively as a series of point images. The resolution obtainable depends on the number of points probed in a feature to be resolved.

Confocal microscopy, especially in the fluorescence mode, is affected by noise of light. In many applications, the number of light quanta (photons) contributing to image formation is extremely small. This is due to the efficiency of the system as a whole and the influencing factors involved, such as quantum yield, bleaching and saturation of fluorochromes, the transmittance of optical elements etc. (see Details “Fluorescence”). An additional influence factor is the energy loss connected with the reduction of the pinhole diameter.

In the following passages, the influences of scanning and noise on resolution are illustrated by practical examples and with the help of a two-point object. This is meant to be an object consisting of two self-luminous points spaced at 0.5 AU (see Details “Optical Coordinates”). The diffraction patterns generated of the two points are superimposed in the image space, with the maximum of one pattern coinciding with the first minimum of the other. The separate visibility of the points (resolution) depends on the existence of a dip between the two maxima (see figure 12).
As a rule, object information is detected by a photomultiplier tube (PMT). The PMT registers the spatial changes of object properties $I(x)$ as a temporal intensity fluctuation $I(t)$. Spatial and temporal coordinates are related to each other by the speed of the scanning process $x = t \cdot v_{\text{scan}}$. The PMT converts optical information into electrical information. The continuous electric signal is periodically sampled by an analog-to-digital (A/D) converter and thus transformed into a discrete, equidistant succession of measured data (pixels) (figure 12).

Types of A/D conversion

The quality of the image scanned depends on the type of A/D conversion which is employed. Two types can be distinguished:

- **Sampling**: The time $(t)$ for signal detection (measurement) is small compared to the time $(T)$ per cycle (pixel time) (see figure 12).
- **Integration**: The signal detection time has the same order of magnitude as the pixel time. Integration is equivalent to an averaging of intensities over a certain percentage of the pixel time known as pixel dwell time. To avoid signal distortion (and thus to prevent a loss of resolution), the integration time must be shorter than the pixel time. The highest resolution is attained with point sampling (the sampling time is infinitesimally short, so that a maximum density of sampling points can be obtained). By signal integration, a greater share of the light emitted by the specimen contributes to the image signal. Where signals are weak (e.g. fluorescence), this is a decisive advantage over point sampling with regard to the signal-to-noise ratio (SNR). Therefore, Carl Zeiss confocal LSM systems operate in the integration mode, as a rule. The absolute integration time can be modified by varying the scanning speed, which also means a change of the pixel time.
Nyquist theorem

It is known from Part 1 that the information content of the signal is limited by the resolving power of the microscope optics. A realistic estimate for the resolving power is the full width at half maximum intensity (FWHMlat) of a point image (see equation 3).

To avoid a loss of information during the scanning process, it is necessary to stick to the Nyquist theorem. The optimal pixel spacing in scanning a periodic signal, as defined by the Nyquist theorem, is half the period of the feature spacing to be resolved, or two pixels per resolvable structure detail. Together with the resolving power defined above, this results in a maximum pixel spacing of 
\[ d_{\text{pix}} = 0.5 \times \text{FWHM}_{\text{lat}} \]

With a two-point object (see explanation on page 17), the pixel spacing needed to separate the two Airy discs in the digitized image is 0.25 AU (figure 13).

If the number of sampling points per feature size is smaller than that given by the Nyquist theorem (undersampling), part of the information will be lost. This is evident in Figure 14c especially by the unresolved fine features.

A greater number of sampling points per feature size (oversampling) means a greater number of readings without a gain in information; simultaneously, the time per pixel becomes shorter. Thus, the volume of data to be processed is inflated, and the noise of the measurement signal increases (see page 20).

Under unfavorable conditions, also artefacts may result out of the digitization process (aliasing). As a rule, this is the case if the feature spacing in the specimen is equal, or nearly equal, to the pixel spacing.
Pixel size

A quantity of decisive importance in this connection is the maximum scanning angle set via the scanning zoom. By varying the scanning angle, it is possible to directly influence the edge length of the scanned field in the intermediate image plane (or object plane), and thus the pixel size (at a given number of pixels per line). The smaller the scanning angle, the smaller is the edge length of the scanned field, and the smaller is the pixel (see the example below).

In this way, the user of a Carl Zeiss confocal LSM can control the sampling rate (pixel size). For setting the suitable scanning zoom for correct Nyquist sampling, the pixel size $d_{\text{pix}}$ in the object plane is important.

For a Carl Zeiss confocal LSM, there is a simple formula, based on the edge length of the scanned field in the intermediate image:

$$d_{\text{pix}} = \frac{\text{system constant}}{\text{number of pixels} \cdot \text{zoom factor} \cdot \text{magnification}_{\text{obj}}^2 \cdot \text{NA}}$$

The minimum scanning zoom needed to fulfill the Nyquist theorem can therefore be calculated as follows:

$$Z \geq \frac{3.92 \cdot \text{NA} \cdot \text{magnification}_{\text{obj}}}{\lambda_{\text{exc}}}$$

For example, with a 40x objective (NA = 1.3), 512 pixels per scan line and a wavelength of 488 nm, the full resolving power (correct sampling) is achieved with a scanning zoom of 4.56 as a minimum; the corresponding pixel size is 95.8 nm. With lower factors of the scanning zoom the pixel size itself will be the limiting factor for resolution (pixel resolution). Higher factors will cause over-sampling. Hence, the zoom factor influences not only the total magnification but also the resolution properties of the system.

With the more recent LSM systems of Carl Zeiss, the number of sampling points can also be influenced by an increase in the number of pixels per scan line.

(The number of pixels (X/Y) per image can be freely selected between 4 x 2 and 2048 x 2048).
The main types of noise important in a confocal LSM system are detector noise (dark noise, secondary emission noise), laser noise, and shot noise of the light (see Details "Sources of Noise"). As a rule, these sources of noise are of a statistical nature. Periodic noise rarely occurs, and if it does, it tends to correlate with defective devices or mechanical vibration in the setup; therefore it has been left out of consideration here.

As the graphs in figure 15 show, the number of photons hitting the PMT depends not only on the intensity of the fluorescence signal (see Details "Fluorescence"), but also on the diameter of the pinhole. The graph shows the intensity distribution of a two-point object resulting behind the pinhole, in normalized (left) and non-normalized form (right). The pinhole diameter was varied between 2 AU and 0.05 AU. At a diameter of 1 AU the pinhole just equals the size of the Airy disk, so that there is only a slight loss in intensity. The gain in resolution, is minimum in this case.

With a pinhole diameter <1AU, resolution improves (better point separation thanks to a deeper dip), which is penalized by a drastic loss in energy. Moreover, it should be considered that it depends on the signal level which noise source dominates. With high-amplitude signals (number of detected photons >10,000), laser noise is the dominating effect, whereas the quality of low signals (number of detected photons <1000) is limited by the shot noise of the light.

Therefore, laser noise tends to be the decisive noise factor in observations in the reflection mode, while shot noise dominates in the fluorescence mode. With recent PMT models (e.g., from Hamamatsu), detector dark noise is extremely low, same as secondary emission noise, and both can be neglected in most practical applications (see Details "Sources of Noise"). Therefore, the explanations below are focused on the influence of shot noise on lateral resolution.

Fig. 15 As shown in Part 1, small pinhole diameters lead to improved resolution (smaller FWHM, deeper dip – see normalized graph on the left). The graphs on the right shows, however, that constraining the pinhole is connected with a drastic reduction in signal level. The drop in intensity is significant from PH <1 AU.
Resolution and shot noise – resolution probability

If the number of photons detected (N) is below 1000, fluorescence emission should be treated as a stochastic rather than a continuous process; it is necessary, via the shot noise, to take the quantum nature of light into account (the light flux is regarded as a photon flux, with a photon having the energy $E = h \nu$). Resolution becomes contingent on random events (the random incidence of photons on the detector), and the gain in resolution obtainable by pinhole constriction is determined by the given noise level. Figure 16 will help to understand the quantum nature of light.

As a possible consequence of the shot noise of the detected light, it may happen, for example, that noise patterns that change because of photon statistics, degrade normally resolvable object details in such a way that they are not resolved every time in repeated measurements. On the other hand, objects just outside optical resolvability may appear resolved because of noise patterns modulated on them. Resolution of the “correct” object structure is the more probable the less noise is involved, i.e. the more photons contribute to the formation of the image.

Therefore, it makes sense to talk of resolution probability rather than of resolution. Consider a model which combines the purely optical understanding of image formation in the confocal microscope (PSF) with the influences of shot noise of the detected light and the scanning and digitization of the object. The essential criterion is the discernability of object details.

Figure 17 (page 22) shows the dependence of the resolution probability on signal level and pinhole diameter by the example of a two-point object and for different numbers of photoelectrons per point object. [As the image of a point object is covered by a raster of pixels, a normalization based on pixels does not appear sensible.]

Thus, a number of 100 photoelectrons/point object means that the point object emits as many photons within the sampling time as to result in 100 photoelectrons behind the light-sensitive detector target (PMT cathode). The number of photoelectrons obtained from a point object in this case is about twice the number of photoelectrons at the maximum pixel (pixel at the center of the Airy disk). With photoelectrons as a unit, the model is independent of the sensitivity and noise of the detector and of detection techniques (absolute integration time / point sampling / signal averaging). The only quantity looked at is the number of detected photons.

Fig. 16: The quantum nature of light can be made visible in two ways:
1. by reducing the intensity down to the order of single photons and
2. by shortening the observation time at constant intensity, illustrated in the graph below. The individual photons of the light flux can be resolved in their irregular (statistical) succession.
A resolution probability of 90% is considered necessary for resolving the two point images. Accordingly, the two-point object defined above can only be resolved if each point produces at least about 25 photoelectrons. With pinhole diameters smaller than 0.25 AU, the drastic increase in shot noise (decreasing intensity of the detected light) will in any case lead to a manifest drop in resolution probability, down to the level of indeterminateness ($\leq 50\%$ probability) at $PH = 0$.

As another consequence of shot noise, the curve maximum shifts toward greater pinhole diameters as the number of photoelectrons drops. The general slight reduction of resolution probability towards greater pinhole diameters is caused by the decreasing effectiveness of the pinhole (with regard to suppression of out-of-focus object regions, see Part 1).

The pinhole diameter selected in practice will therefore always be a trade-off between two quality parameters: noise (SNR as a function of the intensity of the detected light) and resolution (or depth discrimination). The pinhole always needs a certain minimum aperture to allow a minimum of radiation (depending on the intensity of fluorescence) to pass to the detector. Where fluorescence intensities are low, it may be sensible to accept less than optimum depth discrimination so as to obtain a higher signal level (higher intensity of detected light = less noise, better SNR). For most fluorescent applications a pinhole diameter of about 1 AU has turned out to be the best compromise.

---

Fig. 17 The graph shows the computed resolution probability of two self-luminous points (fluorescence objects) spaced at 1/2 AU, as a function of pinhole size and for various photoelectron counts per point object ($e^-$).

The image raster conforms to the Nyquist theorem (critical outer spacing = 0.25 AU); the rasterized image is subjected to interpolation. The photoelectron count per point object is approximately twice that per pixel (referred to the pixel at the center of the Airy disk). Each curve has been fitted to a fixed number of discrete values, with each value computed from 200 experiments.

The resolution probability is the quotient between successful experiments (resolved) and the total number of experiments. A resolution probability of 70% means that 7 out of 10 experiments lead to resolved structures. A probability $> 90\%$ is imperative for lending certainty to the assumption that the features are resolved. If we assume a point-like fluorescence object containing 8 FITC fluorescence molecules (fluorochrome concentration of about 1 nMol) a laser power of 100 µW in the pupil and an objective NA of 1.2 ($n = 1.33$), the result is about 45 photoelectrons / point object on the detection side.
Possibilities to improve SNR

Pinhole diameters providing a resolution probability below 90% may still yield useful images if one uses a longer pixel time or employs the signal averaging function. In the former case, additional photons are collected at each pixel; in the latter case, each line of the image, or the image as a whole, is scanned repeatedly, with the intensities being accumulated or averaged. The influence of shot noise on image quality decreases as the number of photons detected increases. As fluorescence images in a confocal LSM tend to be shot-noise-limited, the increase in image quality by the methods described is obvious.

Furthermore, detector noise, same as laser noise at high signal levels, is reduced. The figures on the right show the influence of pixel time (figure 18) and the influence of the number of signal acquisitions (figure 19) on SNR in [dB]. The linearity apparent in the semilogarithmic plot applies to shot-noise-limited signals only. (As a rule, signals are shot-noise-limited if the PMT high voltage needed for signal amplification is greater than 500 V).

A doubling of pixel time, same as a doubling of the number of signal acquisitions, improves SNR by a factor of $\sqrt{2}$ (3 dB). The advantage of the averaging method is the lower load on the specimen, as the exposure time per pixel remains constant. Photon statistics are improved by the addition of photons from several scanning runs (SNR = $\sqrt{n}$, $N = \text{const}$, $n = \text{number of scans averaged}$). By comparison, a longer pixel time directly improves the photon statistics by a greater number $N$ of photons detected per pixel (SNR = $\sqrt{N}$, $N = \text{variable}$), but there is a greater probability of photobleaching or saturation effects of the fluorophores.

![Variation of pixel time and number of averages](image.png)

Figure 18 and 19: Improvement of the signal-to-noise ratio. In figure 18 (top), pixel time is varied, while the number of signal acquisitions (scans averaged) is constant.
In figure 19 (bottom), pixel time is constant, while the number of signal acquisitions is varied.
The ordinate indicates SNR in [dB], the abscissa the free parameter (pixel time, scans averaged).
The pictures on the left demonstrate the influence of pixel time and averaging on SNR; object details can be made out much better if the pixel time increases or averaging is employed.

Another sizeable factor influencing the SNR of an image is the efficiency of the detection beam path. This can be directly influenced by the user through the selection of appropriate filters and dichroic beamsplitters. The SNR of a FITC fluorescence image, for example, can be improved by a factor of about 4 (6 dB) if the element separating the excitation and emission beam paths is not a neutral 80/20 beamsplitter but a dichroic beamsplitter optimized for the particular fluorescence.

Fig. 20 Three confocal images of the same fluorescence specimen (mouse kidney section, glomeruli labeled with Alexa488 in green and actin labelled with Alexa 564 phalloidin in red). All images were recorded with the same parameters, except pixel time and average. The respective pixel times were 0.8 µs in a), 6.4 µs (no averaging) in b), and 6.4 µs plus 4 times line-wise averaging in c).

1 An 80/20 beamsplitter reflects 20% of the laser light onto the specimen and transmits 80% of the emitted fluorescence to the detector.
Summary

This monograph comprehensively deals with the quality parameters of resolution, depth discrimination, noise and digitization, as well as their mutual interaction. The set of equations presented allows in-depth theoretical investigations into the feasibility of carrying out intended experiments with a confocal LSM.

The difficult problem of quantifying the interaction between resolution and noise in a confocal LSM is solved by way of the concept of resolution probability; i.e. the unrestricted validity of the findings described in Part 1 is always dependent on a sufficient number of photons reaching the detector.

Therefore, most applications of confocal fluorescence microscopy tend to demand pinhole diameters greater than 0.25 AU; a diameter of 1 AU is a typical setting.
Glossary

\[\alpha\] Aperture angle of a microscope objective

\[\text{AU}\] Airy unit (diameter of Airy disc)

\[\text{dpix}\] Pixel size in the object plane

\[\text{FWHM}\] Full width at half maximum of an intensity distribution (e.g. optical slice)

\[n\] Refractive index of an immersion liquid

\[\text{NA}\] Numerical aperture of a microscope objective

\[\text{PH}\] Pinhole; diaphragm of variable size arranged in the beam path to achieve optical sections

\[\text{PMT}\] Photomultiplier tube (detector used in LSM)

\[\text{PSF}\] Point spread function

\[\text{RU}\] Rayleigh unit

\[\text{SNR}\] Signal-to-noise ratio
To give some further insight into Laser Scanning Microscopy, the following pages treat several aspects of particular importance for practical work with a Laser Scanning Microscope.

Pupil Illumination

Optical Coordinates

Fluorescence

Sources of Noise
All descriptions in this monograph suggest a confocal LSM with a ray geometry providing homogeneous illumination at all lens cross sections. The focus generated in the object has an Airy distribution, being a Fourier transform of the intensity distribution in the objective’s pupil plane. However, the truncation of the illuminating beam cross-section needed for an Airy distribution causes a certain energy loss (a decrease in efficiency). In Carl Zeiss microscope objectives, the pupil diameter is implemented by a physical aperture close to the mounting surface.

The Airy distribution is characterized by a smaller width at half maximum and a higher resolving power. Figure 21 (left) shows the intensity distribution at the focus as a function of the truncation factor $T$ (the ratio of laser beam diameter ($1/e^2$) and pupil diameter).

The graph presents the relative intensity distributions at the focus (each normalized to 1) for different truncation factors. (The red curve results at a homogeneous pupil illumination with $T > 5.2$, while the blue one is obtained at a Gaussian pupil illumination with $T \leq 0.5$; the green curve corresponds to a truncation factor $T = 1.3$). The lateral coordinate is normalized in Airy units (AU). From $T = 3$, the Airy character is predominate to a degree that a further increase in the truncation factor no longer produces a gain in resolution. Because of the symmetry of the point image in case of diffraction-limited imaging, the graph only shows the intensity curve in the $+X$ direction. Figure 21 (right) shows the percentage efficiency as a function of pupil diameter in millimeter, with constant laser beam expansion. The smaller the pupil diameter, the higher the $T$-factor, and the higher the energy loss (i.e. the smaller the efficiency).

Example: If the objective utilizes 50% of the illuminating energy supplied, this means about 8% resolution loss compared to the ideal Airy distribution. Reducing the resolution loss to 5% is penalized by a loss of 70% of the illuminating energy. In practice, the aim is to reach an optimal approximation to a homogeneous pupil illumination; this is one reason for the fact that the efficiency of the excitation beam path in a confocal LSM is less than 10%.

The truncation factor $T$ is defined as the ratio of laser beam and pupil diameter of the objective lens used: $T = \frac{d_{\text{laser}}}{d_{\text{pupil}}}$; the resulting efficiency is defined as $\eta = 1 - e^{-\frac{1}{T}}$

The full width at half maximum of the intensity distribution at the focal plane is defined as $FWHM = 0.71 \cdot \frac{\lambda}{NA} \cdot \frac{1}{\sin \theta}$, with $\theta = \sqrt{0.5 + 0.74 \cdot \ln \left( \frac{T}{T_0} \right)}$

With $T < 0.6$, the Gaussian character, and with $T > 1$ the Airy character predominates the resulting intensity distribution.
Optical Coordinates

In order to enable a representation of lateral and axial quantities independent of the objective used, let us introduce optical coordinates oriented to microscopic imaging.

Given the imaging conditions in a confocal microscope, it suggests itself to express all lateral sizes as multiples of the Airy disk diameter. Accordingly, the Airy unit (AU) is defined as:

\[
1 \text{AU} = \frac{1.22 \cdot \lambda}{\text{NA}}
\]

\(\text{NA}\) = numerical aperture of the objective
\(\lambda\) = wavelength of the illuminating laser light

The AU is primarily used for normalizing the pinhole diameter.

Thus, when converting a given pinhole diameter into AUs, we need to consider the system’s total magnification, which means that the Airy disk is projected onto the plane of the pinhole (or vice versa).

Analogously, a sensible way of normalization in the axial direction is in terms of multiples of the wave-optical depth of field. Proceeding from the Rayleigh criterion, the following expression is known as Rayleigh unit (RU):

\[
1 \text{RU} = \frac{1.22 \cdot \lambda}{\text{NA} \cdot n}
\]

\(n\) = refractive index of immersion liquid

\(\text{RU}\) is used primarily for a generally valid representation of the optical slice thickness in a confocal LSM.
Fluorescence is one of the most important contrasting methods in biological confocal microscopy. Cellular structures can be specifically labeled with dyes (fluorescent dyes = fluorochromes or fluorophores) in various ways. Let the mechanisms involved in confocal fluorescence microscopy be explained by taking fluorescein as an example of a fluorochrome. Fluorescein has its absorption maximum at 490 nm. It is common to equip a confocal LSM with an argon laser with an output of 15–20 mW at the 488 nm line. Let the system be adjusted to provide a laser power of 500 µW in the pupil of the microscope objective. Let us assume that the microscope objective has the ideal transmittance of 100%.

With a C-Apochromat 63x/1.2W, the power density at the focus, referred to the diameter of the Airy disk, then is 2.58·10^5 W/cm^2. This corresponds to an excitation photon flux of 6.34·10^23 photons/cm^2 sec. In conventional fluorescence microscopy, with the same objective, comparable lighting power (xenon lamp with 2 mW at 488 nm) and a visual field diameter of 20 mm, the excitation photon flux is only 2.48·10^18 photons/cm^2 sec, i.e. lower by about five powers of ten.

This is understandable by the fact that the laser beam in a confocal LSM is focused into the specimen, whereas the specimen in a conventional microscope is illuminated by parallel light.

The point of main interest, however, is the fluorescence (F) emitted.

The emission from a single molecule (F) depends on the molecular cross-section (σ), the fluorescence quantum yield (Qe) and the excitation photon flux (I) as follows:

\[ F = \sigma \cdot Qe \cdot I \] [photons/sec]

In principle, the number of photons emitted increases with the intensity of excitation. However, the limiting parameter is the maximum emission rate of the fluorochrome molecule, i.e. the number of photons emittable per unit of time. The maximum emission rate is determined by the lifetime (= radiation time) of the excited state. For fluorescein this is about 4.4 nsec (subject to variation according to the ambient conditions). On average, the maximum emission rate of fluorescein is 2.27·10^10 photons/sec. This corresponds to an excitation photon flux of 1.26·10^24 photons/cm^2 sec.

At rates greater than 1.26·10^24 photons/cm^2 sec, the fluorescein molecule becomes saturated. An increase in the excitation photon flux will then no longer cause an increase in the emission rate; the number of photons absorbed remains constant. In our example, this case occurs if the laser power in the pupil is increased from 500 µW to roughly 1 mW. Figure 22 (top) shows the relationship between the excitation photon flux and the laser power in the pupil of the stated objective for a wavelength of 488 nm. Figure 22 (bottom) illustrates the excited-state saturation of fluorescein molecules. The number of photons absorbed is approximately proportional to the number of photons emitted (logarithmic scaling).

| Source: | Handbook of Biological Confocal Microscopy, p. 268/Waggoner |

In the example chosen, \[ F = 1.15 \cdot 10^8 \text{ photons/sec} \] or 115 photons/µsec

The table below lists the characteristics of some important fluorochromes:

<table>
<thead>
<tr>
<th>Absorb. max. (nm)</th>
<th>( \sigma/10^{-16} )</th>
<th>Qe</th>
<th>( \sigma\text{Q}/10^{-16} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rhodamine 594</td>
<td>3.25</td>
<td>0.78</td>
<td>0.91</td>
</tr>
<tr>
<td>Fluorescein 490</td>
<td>2.55</td>
<td>0.71</td>
<td>1.81</td>
</tr>
<tr>
<td>Texas Red 596</td>
<td>3.13</td>
<td>0.51</td>
<td>1.68</td>
</tr>
<tr>
<td>Cy 3.18</td>
<td>4.97</td>
<td>0.14</td>
<td>0.69</td>
</tr>
<tr>
<td>Cy 5.18</td>
<td>7.66</td>
<td>0.18</td>
<td>1.37</td>
</tr>
</tbody>
</table>

The table below lists the characteristics of some important fluorochromes:
What has been said so far is valid only as long as the molecule is not affected by photobleaching. In an oxygen-rich environment, fluorescein bleaches with a quantum efficiency of about \(2.7 \times 10^{-5}\). Therefore, a fluorescence molecule can, on average, be excited \(n = 26,000\) times (\(n = Q/Q_b\)) before it disintegrates.

With \(n = \frac{t}{t_{\text{em}}^\text{max}}\) and referred to the maximum emission rate, this corresponds to a lifetime of the fluorescein molecule of about 115 \(\mu\)s. It becomes obvious that an increase in excitation power can bring about only a very limited gain in the emission rate. While the power provided by the laser is useful for FRAP (fluorescence recovery after photobleaching) experiments, it is definitely too high for normal fluorescence applications. Therefore it is highly important that the excitation power can be controlled to fine increments in the low-intensity range.

A rise in the emission rate through an increased fluorophore concentration is not sensible either, except within certain limits. As soon as a certain molecule packing density is exceeded, other effects (e.g. quenching) drastically reduce the quantum yield despite higher dye concentration. Another problem to be considered is the system’s detection sensitivity. As the fluorescence radiated by the molecule goes to every spatial direction with the same probability, about 80% of the photons will not be captured by the objective aperture (\(\text{NA} = 1.2\)).

With the reflectance and transmittance properties of the subsequent optical elements and the quantum efficiency of the PMT taken into account, less than 10% of the photons emitted are detected and converted into photoelectrons (photoelectron = detected photon). In case of fluorescein (\(\text{NA} = 1.2\), 100 \(\mu\)W excitation power, \(\lambda = 488\) nm), a photon flux of \(4.29 \times 10^{-24}\) results. In combination with a sampling time of 4 \(\mu\)s/pixel this means \(3-4\) photoelectrons/molecule and pixel.

In practice, however, the observed signal will be a labeled cell. As a rule, the cell volume is distinctly greater than the volume of the sampling point. What is really interesting, therefore, is the number of dye molecules contained in the sampling volume at a particular dye concentration. In the following considerations, diffusion processes of fluorophore molecules are neglected. The computed numbers of photoelectrons are based on the parameters listed above.

With \(\lambda = 488\) nm and \(\text{NA} = 1.2\) the sampling volume can be calculated to be \(V = 1.27 \times 10^{-18}\) l. Assuming a dye concentration of 0.01 \(\mu\)Mol/l, the sampling volume contains about 80 dye molecules. This corresponds to a number of about 260 photoelectrons/pixel. With the concentration reduced to 1 nMol/l, the number of dye molecules drops to 8 and the number of photoelectrons to 26/pixel.

Finally it can be said that the number of photons to be expected in many applications of confocal fluorescence microscopy is rather small (<1000). If measures are taken to increase the number of photons, dye-specific properties such as photobleaching have to be taken into account.

---

**Fig. 22** Excitation photon flux at different laser powers (top) and excited-state saturation behavior (absorbed photons) of fluorescein molecules (bottom).
Sources of Noise

Sources of noise effective in the LSM exist everywhere in the signal chain – from the laser unit right up to A/D conversion. Essentially, four sources of noise can be distinguished:

**Laser noise q**
Laser noise is caused by random fluctuations in the filling of excited states in the laser medium. Laser noise is proportional to the signal amplitude $N$ and therefore significant where a great number of photons ($N < 10000$) are detected.

**Shot noise (Poisson noise)**
This is caused by the quantum nature of light. Photons with the energy $h\nu$ hit the sensor at randomly distributed time intervals. The effective random distribution is known as Poisson distribution. Hence,

$$\text{SNR} = \frac{\Delta N_{\text{noise}}}{\sqrt{N}}$$

where $N = \text{number of photons detected per pixel time}$ ($\sim$ photoelectrons = electrons released from the PMT cathode by incident photons). With low photoelectron numbers ($N < 1000$), the number $N$ of photons incident on the sensor can only be determined with a certainty of $\pm \sqrt{N}$.

$N$ can be computed as

$$N = \frac{\text{photons}}{\text{QE}(\lambda)} \cdot \text{pixel time}$$

where $\text{QE}(\lambda) = \text{quantum yield of the sensor at wavelength } \lambda$.

$1\text{ photon} = h/\lambda$, $c = \text{light velocity}$; $h = \text{Planck's constant}$

**Secondary emission noise**
Caused by the random variation of photoelectron multiplication at the dynodes of a PMT. The amplitude of secondary emission noise is a factor between 1.1 and 1.25, depending on the dynode system and the high voltage applied (gain). Generally, the higher the PMT voltage, the lower the secondary emission noise; a higher voltage across the dynodes improves the collecting efficiency and reduces the statistical behavior of multiplication.

**Dark noise**
Dark noise is due to the generation of thermal dark electrons $N_d$, irrespective of whether the sensor is irradiated. $N_d$ statistically fluctuates about $\sqrt{N_d}$. Dark noise is specified for a PMT voltage of 1000 V; with lower voltages it progressively loses significance. Dark noise can be reduced by cooling the sensor. However, the reduction is significant only if $N_d \leq N_d$, e.g. in object-free areas of a fluorescence specimen. In addition, the dark noise must be the dominating noise source in order that cooling effects a signal improvement; in most applications, this will not be the case.

Additional sources of noise to be considered are amplifier noise in sensor diodes and readout noise in CCD sensors. In the present context, these are left out of consideration.

The mean square deviation $\Delta N$ from the average $(N + N_d)$ of the photoelectrons and dark electrons registered, so that the total signal-to-noise ratio can be given as

$$\text{SNR} = \frac{N^2}{\text{se}^2 (N+N_d) (1+q^2)}$$

where

$N = \text{number of photons detected per pixel time}$ ($\sim$ photoselectrons = electrons released from the PMT cathode by incident photons).

$\text{se} = \text{multiplication noise factor of secondary emission}$

$q = \text{peak-to-peak noise factor of the laser}$

$N_d = \text{number of dark electrons in the pixel or sampling time}$

Example:
For $N = 1000, N_d = 100, \text{se} = 1.2$, and $q = 0.05$

$$\text{SNR} = \sqrt{\frac{1000^2}{1.2^2 (1000+100) (1+0.05^2)}} = 25.1$$
LITERATURE


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We gratefully acknowledge the assistance of many other staff members who contributed to this brochure.

Subject to change.

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Subject to change.
The Confocal Laser Scanning Microscope

Carl Zeiss · 07740 Jena · Germany · E-mail: micro@zeiss.de · www.zeiss.de/lsm

3 Steps to Get a Confocal Image

1. View specimen in VIS mode
   Focus the specimen in epi-fluorescence mode using the binocular and center the field of view. Select fluorescence filter cube according to application (e.g., FITC or Cy3) via SW (window “Microscope Control”).
   Match the field of view to application (e.g., FITC or Cy3) via SW (window “Microscope Control”).
   Select fluorescence filter cube according to application (e.g., FITC or Cy3) via SW (window “Microscope Control”).

2. Load an LSM configuration
   Activate LSM mode (operate manual tube slider or button “(LSM)”). Open window “Configuration control”, and select predefined configuration from list (Single Track).
   A click on “Apply” automatically sets up the system: laser lines, attenuation, emission filters, beamsplitters (HFT, NFT), pinhole diameter, detector settings (channels, gain, offset). Click on “Reset” button (detailed image/database window) to restore settings of a previous experiment.

3. Scan an image
   Click on “Find” button (right row in window “Scan Control”) => System automatically quasi images (window, optimizes detector settings (matches PMT gain and offset to dynamic range of 8 or 12 bit), and scans an image.
   See operation manual for scanning a stack of slices, time series etc.

How to Enhance Image Quality

More signal!
- Change to longer pixel dwell times by reducing scanning speed
- Use “Average” method: calculation of “Sum” or “Mean” value of pixels of consecutive “Line” or “Frame” scans
- Increase bandwidth of emission filter (e.g., LP instead of BP)
- Enlarge pinhole diameter; note: optical slice thickness increases accordingly
- Increase excitation energy (laser power), but pay attention to bleaching, saturation and phototoxic effects.

More details!
- Use objective with higher numerical aperture (NA): x/y-resolution = \( \lambda / (2 \cdot NA^2) \)
- Increase “FrameSize” = number of pixels per line * lines per frame, e.g., 1024 x 2048 or 2048 x 2048 (min. 4 x 2).
- Optimize scan zoom (Z), i.e., pixel size ≤ 0.25 x diameter of Airy disk (e.g., Objective 40x, NA 1.3, 1 x 480 or 2 x 480).
- Increase dynamic range (change from 8 to 12 bit per pixel).

More reliability!
- Use Multitracking: very fast switching of excitation wavelengths; prevents crosstalk of signals between channels; predefined configurations available.
- Use ROI (Region Of Interest) function: significantly reduces excited area of specimen and increases acquisition rate close to constant SNR; several ROIs of any shape can be defined and used simultaneously.

Photomultiplier (PMT)
- Detector: pixel-wise detection of photons emitted/ reflected by the respective specimen detail
- Parameters: “Detector Gain” a PMT high voltage, “PMT Offset” dark level setting, “Amplifier Gain” + electron preamplification
- Calibration: “Amplifier Offset” on image background (single pixel area). “Detector Gain” according to scanned image (object), setting aid = “Range Indicator” (“Y-axis”), goal: least number of overmodulated (red), Gain and undermodulated (blue), Offset pixels

Confocal Pinhole
- Depth discrimination = confocal aperture to prevent detection of out-of-focus light (optical sectioning)
- Diameter: determines thickness of optical slice; optimum selection = 1 Airy unit = best trade-off between depth discrimination capability and efficiency
- Axial resolution: factory-adjusted for all beam path configurations; can be modified manually (= “Maintain-Pinhole”)

Confocal Laser Scanning Microscope

Carl Zeiss
07740 Jena · Germany · E-mail: micro@zeiss.de · www.zeiss.de/lsm

Microscopy from Carl Zeiss

Laser
- Light source – projected into specimen
- Laser power: adjustable via attenuation device
- AOTF, AOJF and tube current setting (A)
- Attenuation: influenced by using lower tube current.
- Degree of slice will be increased (β = minimum ratio
- Scanning mode: protons laser light not suitable for image acquisition
- Laser line: can be chosen via selection device (AOTF, AOJF)
- Independent on fluorescent dyes. Generally, the shorter the wavelength, the higher the resolution

Application goals:
1. Protect specimen (reduction of dye bleaching and phototoxic effects) by reduction of laser power.
2. Maximize fluorescence signal (higher SNR) by longer pixel dwell times or averaging

Resolution: maximum resolution can be achieved if pixel size is set correctly (at least 4 x 4 pixels (x, y) per specimen line by line

Pixel size = wavelength, the higher the resolution

Pixel time: influences SNR of image; the longer the pixel time,

Laser source

Autofocus

Optical image formation
- “Amplifier Offset” = black level setting, “Amplifier Gain” + amplifier postamplification
- “Palette” = combination of main (HFT) and secondary (NFT) dichroic mirrors and emission filters BFP & WBP (red, blue, blue, blue, blue, blue, blue, blue)
- “Offset” = effects spectral division of (different) fluorescence emissions (e.g., NFT 545: reflects light of λ > 545nm and transmits light of λ < 545nm)
- LP 505P determines bandwidth of fluorescence emission for the respective channel (e.g., LP 505: λ < 505 nm – detection)

Objective Lens
- Optical image formation = determines properties of image quality such as resolution (y, z)
- Numerical Aperture (NA): determines imaged spot size (given with wavelength), and substantially influences the minimum optical slice thickness achievable
- Airy disk index (NA = 1.4): determines imaged spot size
- NFT: effects spectral division of (different) fluorescence emissions (e.g., NFT 545: reflects light of λ > 545nm and transmits light of λ < 545nm)
- HFT: separates excitation and emission light
- LP 505P determines bandwidth of fluorescence emission for the respective channel (e.g., LP 505: λ < 505 nm – detection)

Objective Pinhole
- Optical image formation = determines properties of image quality such as resolution (y, z)
- Airy disk index (NA = 1.4): determines imaged spot size
- NFT: effects spectral division of (different) fluorescence emissions (e.g., NFT 545: reflects light of λ > 545nm and transmits light of λ < 545nm)
- HFT: separates excitation and emission light
- LP 505P determines bandwidth of fluorescence emission for the respective channel (e.g., LP 505: λ < 505 nm – detection)

Beam Splitter
- Optical Image formation = determines properties of image quality such as resolution (y, z)
- Airy disk index (NA = 1.4): determines imaged spot size
- NFT: effects spectral division of (different) fluorescence emissions (e.g., NFT 545: reflects light of λ > 545nm and transmits light of λ < 545nm)
- HFT: separates excitation and emission light
- LP 505P determines bandwidth of fluorescence emission for the respective channel (e.g., LP 505: λ < 505 nm – detection)

Optical Image formation = determines properties of image quality such as resolution (y, z)
- Numerical Aperture (NA): determines imaged spot size (given with wavelength), and substantially influences the minimum optical slice thickness achievable
- Airy disk index (NA = 1.4): determines imaged spot size
- NFT: effects spectral division of (different) fluorescence emissions (e.g., NFT 545: reflects light of λ > 545nm and transmits light of λ < 545nm)
- HFT: separates excitation and emission light
- LP 505P determines bandwidth of fluorescence emission for the respective channel (e.g., LP 505: λ < 505 nm – detection)
Confocal Laser Scanning Microscopy

Applications in Research and Teaching. Design, Functions, Methods.
Having decoded the human genome, biomedical research today is focused on exploring the interaction between cellular components. Scientists want to find out which protein is where, and at what time, and what other structural and functional modules it interacts with.

In the search for answers to these questions, imaging systems based on the classical light microscope have come to play an unprecedented role, thanks to many technical innovations and a high degree of automation. Many experiments have only become possible because of the new functions provided by modern microscopes.
Confocal laser scanning microscopes (LSMs) are distinguished by their high spatial and temporal resolving power. They clearly outperform classical light microscopes especially by their axial resolution – a quality that enables users to acquire optical sections (slices) of a specimen. An object can thus be imaged completely in three dimensions and subsequently visualized as a 3D computer image. In another group of applications, exactly defined areas of a specimen can be selectively illuminated by laser light. This functionality is essential for quantitative investigations of dynamic processes in living cells using techniques such as FRAP (fluorescence recovery after photobleaching), FRET (fluorescence resonance energy transfer), photoactivation and photoconversion.

New acquisition methods on the LSM permit the detection of additional properties of the emitted light including spectral signatures and fluorescence lifetimes. With such information it is possible to increase the number of fluorescent labels used in an experiment, or to use fluorochrome combinations unthinkable with conventional detection methods. The advantages are obvious: the more components in a cellular process that are observed simultaneously, the greater the yield of information.

Because of its versatility, laser scanning microscopy has become an established mainstream method in biomedical research – a tool permitting scientists to follow innovative experimental paths. This article will show which basic functions and applications of laser scanning microscopy can be taught in academic tuition. The modern method of confocal laser scanning microscopy can be taught on the basis of classical light microscopy, an established part of fundamental biomedical teaching.

*In the non-confocal image, the interesting information of the focal plane mixes with unwanted information from extrafocal specimen planes; differently stained details result in a color mix.*

*In the confocal image, object details blurred in the non-confocal image are visible clearly and in greater contrast.*
The Confocal Principle

In this chapter, the mode of operation of an LSM will be explained using a fluorescence-labeled specimen as an example. Fluorescent dyes, also known as fluorochromes, are used as markers in most biomedical applications to make the structures of interest visible. But laser scanning microscopes can just as well be combined with other microscopic contrast techniques such as reflected light or polarization.

An LSM can be easily understood as a modified light microscope supplemented by a laser module that serves as a light source, and a scanning head (attached to the microscope stand) that is used to detect the signal. Signal processing is effected by an electronic system contained in a box. The whole system is controlled by a computer.

To generate a confocal LSM image, let us first excite the fluorescence marker in a defined specimen area with a laser. For this purpose, monochromatic light from the laser module is coupled into the scanning head via a fiber optic. In the scanning head, the beam is made parallel by means of a collimator, and reflected into the microscope’s light path by the principal dichroic beam splitter. The objective focuses the excitation beam onto a small three-dimensional specimen region called the excitation volume. The spatial extension of this volume is directly related to the system’s resolving power. The greater the numerical aperture of the objective, the smaller the focal volume, and the higher the resolution. The position of the excitation volume can be shifted laterally (in X and Y) by means of two scanning mirrors, and vertically (in Z) with the microscope’s focusing knob. The current Z position marks the system’s focusing plane.
The laser light illuminating a particular detail of the specimen in order to excite fluorescence is focused by the objective into the focal plane. Fluorescence excitation and emission are most efficient within the focal volume. Therefore, the fluorescence from labeled structures in the focal plane forms a sharp image. The laser light, whilst less efficient, is still intensive enough to also excite fluorescently labeled structures above and below the focal plane. Light emitted there would be superimposed onto the sharp focal plane image and blur it. This is prevented by a pinhole diaphragm arranged in the ray path, which only permits light emitted in the focal plane to reach the detector.

The pinhole is essential to the generation of sharp images and for the optical sectioning capability. The very designation of confocal laser scanning microscopy refers to the pinhole, as this is in a plane conjugated to that of the focal plane (conjugate plane). The thickness or Z dimension of an optical section can be set by motor-driven adjustment of the pinhole diameter. Fluorescence light from the focal plane, having passed the pinhole, is then detected by a photomultiplier. As an LSM image is formed sequentially, i.e. pixel by pixel, the detector does not require any spatial resolution. It merely measures the fluorescence intensity as a function of time. The image proper is formed only when the intensity measured by the detector is assigned to the corresponding site of the laser focus in the specimen. The laser beam is directed by the two independent scanning mirrors to scan the specimen in a line-by-line mode. The result of the scanning process is an XY image that represents a two-dimensional optical section of the specimen.
Two-Dimensional Images

For examining flat specimens such as cell culture monolayers, it is usually sufficient to acquire one XY image to obtain the desired information. The same applies if the specimen is a three-dimensional tissue section of which a single optical section is representative.

The thickness of the optical section (slice) and the focal position are selected so that the structures of interest are contained in the slice. The lateral resolution of a 2D image is defined by the pixel size in X and Y. The pixel size, in turn, varies with the objective used, the number of pixels per scan field, and the zoom factor. Pixels that are too large degrade resolution, whereas pixels too small require longer scanning times, bleach the specimen and generate superfluous data volumes. The optimum pixel size for a given objective and a given zoom factor can be set by selecting the number of pixels with a mouse click.

The procedure for a two-dimensional image:

1. Position and focus on the specimen in the Vis(ual) mode
2. Select the configuration to match the fluorochromes used
3. Define pixel resolution, scanning speed and, where required, Average Mode
4. Set the optical slice thickness by means of the pinhole diameter
5. Adapt the dynamic range to the specimen; automatically via Find, or manually via Gain and Offset
6. Adapt the scanning field to specimen substructures, using the Crop function
Confocal section through the cerebellum of a rat.
Green: astroglia cells (GFAP labeling); red: superoxide dismutase in neurons.

Double labeling of a Drosophila retina.
Green: actin; red: Crumbs.
Specimen: Dr. O. Baumann, University of Potsdam, Germany.
Three-Dimensional Images

To record the three-dimensional structure of a specimen, several two-dimensional optical sections are made in different focal planes. The result is an XYZ image stack, which can be visualized, processed and analyzed.

The optical section is selected by shifting the position of the focus in the specimen. This can be effected by moving either the objective or the specimen stage along the Z axis, according to the microscope stand design. Whether the image acquisition exhausts the resolving power given by the objective’s numerical aperture depends on the thickness of the optical slice and on the spacing of two successive sections (the Z interval). According to the Nyquist criterion, the optimum Z interval is equal to half the optical slice thickness. If the pinhole diameter is selected to equal one Airy unit (1 AU), an optimum compromise between contrast and intensity is achieved for the XY image. The respective settings can be made by a mouse click in the software.

If the sample to be examined is labeled with more than one fluorochrome, it is necessary to adjust the optical slice thicknesses of the various image channels. The slice thickness is a function of the numerical aperture of the objective, the wavelength used, and the pinhole diameter. It differs for channels detecting light of different wavelengths. In the systems of the Zeiss LSM 510 family, every detector is equipped with a separate pinhole. This makes it easy to equalize the optical slice thicknesses in the software – an important condition for 3D colocalization analyses or for reconstructing 3D images.

Once a 3D stack of images has been recorded, the user has various presentation options. The data may be displayed as a gallery of depth-coded images or as orthogonal projections of the XY, XZ and YZ planes. To create a 3D impression on a 2D monitor, animations of different viewing angles versus time, shadow projections, and surface rendering techniques are possible.

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The procedure for a three-dimensional image

1. Optimize the recording conditions for an XY image at the center of the three-dimensional specimen (see box for 2D images)
2. Define the Start and Stop stack limits in the Z Setting menu
3. Define the optimum Z interval in the Z Slice menu
4. Acquire the Z stack
5. Display and analyze the stack in one of the Gallery, Ortho or Cut display modes
6. With multiple-labeled specimens, equalize the optical slice thicknesses in the Z Slice menu
Three-dimensional specimen

XYZ image stack of a pollen grain. A series of XY images acquired in different focus positions represents the Z dimension of the specimen.

The orthogonal projection of the three-dimensional image data stack permits the raw data stack to be sectioned anywhere in any of the three mutually perpendicular planes.

Bottom left (above): Horizontal section through the center of the pollen grain (XY image).

Top: Projection of a vertical section along the horizontal axis in the XY image.

Bottom right: Vertical section along the vertical axis in the XY image.

Surface-rendered projection of the pollen grain.
Dynamic processes in living specimens can be recorded by means of time series. Data thus acquired can be analyzed “off-line”, i.e. after image acquisition, or “on-line”, i.e. right during the experiment, for example in the Online Ratio mode.

Time series are defined by a start time and the time interval between two successive images. The series can be started by a mouse click, automatically at a preselected time, or by some external trigger. To analyze a time series, the Physiology software option allows fluorescence intensity changes to be quantified in defined regions of interest (ROIs).

Within a time series, the LSM 510 permits selective, point-accurate illumination of ROIs with laser light. This function is useful for generating a photobleaching routine within a FRAP experiment (fluorescence recovery after photobleaching), for analyzing dynamic processes, and for the photoactivation or photoconversion of suitable fluorochromes. Complex time series experiments, with different images to be taken at different sites within a specimen according to a defined time pattern, can be defined by means of a special software option.

The procedure for a time series

1. Define the image dimensions to be recorded versus time (XY image, Z stack, λ stack)
2. Optimize the recording conditions at minimum laser output to avoid or minimize bleaching
3. Define the number of images to be taken and the time interval between two successive images (Time Interval or Time Delay)
4. Combine with a photobleaching routine if required: define the region to be bleached, the laser line and its power, the number of bleaching actions, and the bleaching start time within the series
5. Start the time series with the Start button, at a preselected time, or by an external trigger
Investigation of protein movements

Time series of PKC-GFP transfected HeLa cells.
The stimulation of the cells with PMA at the time $t=1$ min leads to a redistribution of PKC from the cytosol to the cytoplasmic membrane (times in minutes).
Specimen: Dr. S. Yamamoto,
Medical University of Hamamatsu, Japan.

Evaluation of the experiment
Selection of ROIs within the specimen.
ROI 1: Cytosol
ROI 2: Cytoplasmic membrane

The individual intensities (upper graph) and the ratio of intensities in the two ROIs marked in the top picture (lower graph) illustrate PKC redistribution. Colors are assigned correspondingly.
Multifluorescence – The Crosstalk Problem and Its Solution

If a specimen is labeled with more than one fluorochrome, each image channel should only show the emission signal of one of them.

If, in a specimen labeled red and green, part of the green light is detected in the red channel, the phenomenon is known as crosstalk or bleed-through. This may lead to misleading results, especially in colocalization experiments.

One can distinguish between two kinds of crosstalk: emission and excitation crosstalk.

In a pure emission crosstalk between two fluorochromes A and B, the two emission spectra will overlap, but the laser lines will excite the dyes independently of each other; i.e. there is no overlap of the excitations. Excitation crosstalk would occur if the laser that excites fluorochrome A also partially excited fluorochrome B.

The problem of emission crosstalk can be solved by sequential excitation and detection (Multitracking) of the fluorochromes. In case of a combination of excitation and emission crosstalk, additional spectral information is needed for separating the emission signals.

Emission crosstalk
Section through a mouse kidney, double-labeled with Alexa 488 (wheat germ agglutinin) and Alexa 568 (phalloidin). Simultaneous excitation with 488 and 543 nm. The emission of Alexa 488 is detected in both the green (BP 505-530 nm) and red (BP 560-615 nm) channels. Because of this bleed-through, the areas labeled with Alexa 488 appear yellow in the superposition and could be misinterpreted as colocalization with the Alexa 568.

Elimination of emission crosstalk by Multitracking
If Alexa 488 and 568 are excited and detected sequentially, no green signal is detected in the red channel. Structures labeled with Alexa 488 appear green in channel superposition.
Emission crosstalk of Alexa Fluor 488 and 546

The excitation efficiency of the two fluorochromes is determined by the point of intersection between the laser line used and the excitation spectrum (dotted line).

Accordingly, Alexa Fluor 488 is excited to about 80%, Alexa Fluor 546 to about 60%.

At a level of about 5%, the excitation spectrum of Alexa Fluor 546 is also intersected by the 488 nm laser line (arrow). Theoretically, this constitutes excitation crosstalk, as one line excites both markers, but it is inefficient enough to be negligible.

Contrary to this, the emission spectra of the two dyes overlap significantly.

The red area marks the emission crosstalk of Alexa Fluor 488 occurring if Alexa Fluor 546 is detected to the right of the 543 nm laser line.

Combined excitation and emission crosstalk

If GFP is used together with YFP, the emission spectra will overlap considerably.

The red area marks the emission crosstalk between GFP and YFP occurring if YFP is detected to the right of the 514 nm laser line.

In addition, there will be a pronounced excitation crosstalk. The 488 nm line excites not only GFP but also YFP to an efficiency of about 30% (arrow).

Source: http://home.ncfcrf.gov/ccr/flowcore/welcome.htm; modified
The acquisition of spectral data becomes necessary where the overlapping emission signals of multiple-labeled specimens have to be separated, or where the cellular parameter to be measured is coded by changes of the emission spectrum (e.g., FRET and ratio imaging of ion concentrations).

The LSM 510 META is a system for the fast acquisition of images of high spectral resolution. The hardware enabling this functionality consists of a spectrally dispersive element, a photomultiplier (PMT) with 32 parallel detection channels (META Detector), and special electronic circuitry for detector control and signal amplification.

Whereas the beam paths for conventional and META detection are identical on the excitation side, the emission beam for spectral detection, after having passed the pinhole, hits a reflective grating. The grating spreads the beam into a spectrum and projects it onto the surface of the linear detector array. Each of the 32 PMT elements in that array thus registers a different part of the spectrum, each part having a width of 10 nm. The result is a lambda stack of XY images in which each image represents a different spectral window.

The procedure for a lambda stack

1. Select the spectral range in the Lambda-Mode
2. Activate the excitation laser lines
3. Carefully control the dynamic range to avoid over- and underexposed pixels (Range Indicator)
4. In multiple-labeled specimens, vary the power of the respective laser lines instead of the Amplifier Gain, in order to match the signal intensities of the fluorochromes
5. Record the lambda stack
6. Display the data in one of the modes: Gallery, Single, Slice, Max or λ-coded
By connecting adjacent detector elements (binning), the spectral width of the images can be extended. From a lambda stack, the intensity of the signal for each pixel of the image can be extracted as a function of wavelength. These spectral "fingerprints" can easily be obtained for any image area by means of the Mean of ROI function. Lambda stacks can be recorded as time series, Z stacks, or as Z stacks versus time. In the last-named case, the result would be a five-dimensional image file with the coordinates, X, Y, Z, lambda and time.

The META Detector is good not only for recording lambda stacks, but also as a channel detector in the conventional mode. By binning the respective detector elements in this mode, the optimum spectral bandwidth can be adjusted for any fluorescent dye.
Emission Fingerprinting

Emission Fingerprinting is a method for the complete separation (unmixing) of overlapping emission spectra. It is used with specimens labeled with more than one fluorescent dye, exhibiting excitation and emission crosstalk.

The typical raw data for Emission Fingerprinting are lambda stacks. The previous chapter described how they are recorded by means of the META Detector. The second step is to define reference spectra for all spectral components contained in the specimen. As a rule, these are dyes internationally used for labeling the specimen. Other possible components are autofluorescent and highly reflecting structures. Autofluorescences, in particular, often have rather broad emission spectra that overlap with the fluorescent markers; this makes them an added source of “impurities” degrading the signals in conventional laser scanning microscopy.

With Emission Fingerprinting, autofluorescences are simply included in the unmixing process. The user can subsequently decide between switching the autofluorescence channel off and using it to obtain structural information possibly contained in the specimen.

The reference spectra can either be loaded from a spectra database, or directly extracted from the lambda stack. For the latter version, the user has two options. One is to define spectra via ROIs. The other uses a statistical method, Automatic Component Extraction (ACE), to find the reference spectra. In either case, the images of the lambda stack must contain structures marked with a single fluorochrome only.

The third step of Emission Fingerprinting is Linear Unmixing, which converts the lambda stack into a multichannel image. Each spectral component of the specimen is then displayed in one channel only. The accuracy of the technique allows the complete unmixing even of such dyes whose spectra have almost identical emission maxima.

Linear Unmixing

Linear mathematical algorithm for spectral unmixing. If we regard a pixel of a lambda stack that represents a locus in the specimen where three fluorescent dyes A, B and C with their spectra $S(\lambda)_dye A$, $S(\lambda)_dye B$ and $S(\lambda)_dye C$ overlap, the cumulative spectrum $\Sigma S(\lambda)$ measured can be expressed as

$$\Sigma S(\lambda) = [\text{intensity} \cdot S(\lambda)]_{dye A} + [\text{intensity} \cdot S(\lambda)]_{dye B} + [\text{intensity} \cdot S(\lambda)]_{dye C}$$

By means of known reference spectra $S(\lambda)_dye A$, $S(\lambda)_dye B$ and $C$, the equation can be solved for the intensities of the dyes A, B and C, which yields the intensity shares of the three dyes for this pixel. If this calculation is made for each pixel, a quantitatively correct 3-channel image results, in which each channel represents a single dye.
The 3 Steps of Emission Fingerprinting

1 Recording of a lambda stack
The illustration shows an 8-channel image of a cell culture transfected with GFP and YFP. Each image shows the mean wavelength of the channel.

2 Definition of reference spectra
The reference spectra were obtained by means of lambda stacks of cells single-marked with GFP and YFP, respectively. Top: Lambda-coded projections of a cell marked with GFP (left) or YFP (right). Bottom: Reference spectra for GFP (green) and YFP (red).

3 Linear Unmixing
Using the reference spectra from the lambda stack, the Linear Unmixing function generates a two-channel image, in which each channel represents only one of the two fluorochromes. Top: GFP Center: YFP Bottom: Both channels superimposed
Channel Unmixing

If the emission spectra of fluorescent markers overlap only slightly, the signals can be separated with the Channel Unmixing function.

As raw data for unmixing in this case, it is sufficient to have multichannel images in which one of the marker dyes dominates in each channel. Such images can be acquired without the META Detector, i.e. with an LSM PASCAL, LSM 510 or a CCD camera.

Channel Unmixing also allows unmixing based on the excitation behavior of dyes, if the raw data are multichannel images in which the channels differ only by their excitation wavelength.

Double labeling of the nervous system of a zebrafish embryo
Two-channel single-track images with emission crosstalk. The nerve labeled with Alexa 488 can be discerned (arrows) in the green (top) and, faintly, in the red channel (center). Bottom: Superposition of the two channels.

The same images after Channel Unmixing. The Alexa 488-positive nerve is visible in the green channel (top) only but vanished from the red one (Center). Bottom: Superposition of the two channels.

Specimen: Prof. M. Bastmeyer, Friedrich Schiller University of Jena, Germany.
Online Fingerprinting

The functionality of Online Fingerprinting can be used to separate overlapping emissions even while a time series is being recorded. This may be of decisive importance where dynamic processes are investigated.

Here, a reference spectrum is assigned to each image channel before image acquisition starts. During the experiment proper, lambda stacks are acquired and immediately unmixed in a background operation. The user sees the unmixed multichannel image during the acquisition of the time series. Online Fingerprinting is of advantage especially in spectral FRET experiments and in studies of dynamic processes with fluorescent proteins.

Visualization of FRET by means of acceptor photobleaching

Expression of a FRET-positive protein construct (CFP linker citrin) in HEK 293 cells.

Recording conditions: Simultaneous excitation with 458 and 514 nm.
Spectral detection from 462 to 655 nm in Lambda Mode.

Online Fingerprinting and simultaneous display of the two-channel image (CFP blue, citrin green).
During the combined time- &-bleaching series (bleaching region marked), citrin (green channel) as a FRET partner is destroyed by intensive irradiation with 514 nm. The decrease in FRET is visible as an increase in CFP fluorescence (blue channel).

Specimen: PD Dr. M. Schäfer, Charité University Hospital, Berlin, Germany
Spectral Imaging

Excitation Fingerprinting

By means of tunable excitation lasers such as those used in multiphoton systems, it is possible to detect also the excitation spectra of fluorochromes. These can be used for unmixing as an alternative to emission spectra.

Multiphoton systems are a special class of confocal laser scanning microscopes, distinguished from classical one-photon systems essentially by an additional light source, known as a multiphoton or NLO (non-linear optics) laser.

The infrared (IR) light emitted by such lasers can penetrate tissues to greater depths than visible light can. Due to its low phototoxicity, IR light is suitable for long-time observation of live samples. Usually, the emission wavelength of these lasers can be varied continuously to excite the respective fluorochrome used in the multiphoton mode. In Excitation Fingerprinting, this property is used for the acquisition of excitation lambda stacks. For that purpose, the multiphoton laser is controlled by the LSM software to shift its excitation wavelength by a defined interval before every new image. The image stacks thus recorded can be used for the unmixing of spectral components differing by their excitation properties, analogously to the (emission) lambda stacks described before. For more information on multiphoton microscopy, refer to the literature cited on the rear cover.

The procedure for Excitation Fingerprinting

1. Define an excitation lambda stack (wavelength range and interval size) in the Excitation Fingerprinting macro

2. Record the excitation lambda stack

3. Define the reference spectra via single-labeled specimen regions, single-labeled reference samples, or by using the ACE function

4. Run Linear Unmixing
Excitation Fingerprinting separates widely overlapping emission signals by their excitation spectra.

Retina of a Drosophila fly, labeled for actin (Alexa Fluor 586 phalloidin); autofluorescence and emission signal can be cleanly separated by Emission Fingerprinting.

Specimen: PD Dr. O. Baumann, University of Potsdam, Germany
Laser scanning microscopy has become a mainstream technique in biomedical research. Thorough familiarity with its theoretical principles and application know-how is a prerequisite for successful experimentation.

Carl Zeiss offers seminars that teach the fundamental theory and explain biomedical research methods, followed by intensive practical hands-on training in small groups. The participants in these courses have access to various combinations of microscopes and latest generation LSM systems made by Carl Zeiss. There is no better way to efficiently acquire new know-how and skills in using up-to-date LSM equipment.

For details, see www.zeiss.de/courses
Modern laser scanning microscopes are versatile tools for visualizing cellular structures and analyzing dynamic processes in biomedical research.

Apart from mere imaging, Carl Zeiss laser scanning microscopes are designed for the quantification and analysis of image-coded information. Among other things, they allow easy determination of fluorescence intensities, distances, areas and their changes over time. The LSM 510 META, in particular, is capable of quickly detecting and quantitatively unmixing the spectral signatures of fluorescent dyes that closely resemble each other.

Many software functions analyze important parameters such as the degree of colocalization of labeled structures, or the ion concentration in a specimen.

With their capabilities for acquiring, evaluating and presenting experimental data, LSM systems made by Carl Zeiss are tailored to the requirements of scientists of today and tomorrow.

Summary
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LINKS

Carl Zeiss website on contrasting techniques in light microscopy www.zeiss.de/contrasts

EAMNET website on FRAP www.embl-heidelberg.de/eamnet/html/teaching_modules.html

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