The DCS-120 Confocal Scanning FLIM System
An Overview

Abstract: The DCS-120 system uses excitation by ps diode lasers or femtosecond titanium-sapphire lasers, fast scanning by galvanometer mirrors, confocal detection, and FLIM by bh’s multidimensional TCSPC technique to record fluorescence lifetime images at high temporal resolution, high spatial resolution, and high sensitivity [1]. The DCS-120 system is available with inverted microscopes of Nikon, Zeiss, and Olympus. It can also be used to convert an existing conventional microscope into a fully functional confocal or multiphoton laser scanning microscope with TCSPC detection. Due to its fast beam scanning and its high sensitivity the DCS-120 system is compatible with live-cell imaging. DCS-120 functions include simultaneous recording of FLIM or steady-state fluorescence images simultaneously in two fully parallel wavelength channels, laser wavelength multiplexing, time-series FLIM, Z stack FLIM, phosphorescence lifetime imaging (PLIM), fluorescence lifetime-transient scanning (FLITS) and FCS recording. Applications focus on lifetime variations by interactions of fluorophores with their molecular environment. Typical applications are ion concentration measurement, FRET experiments, autofluorescence imaging, and plant physiology.

Architecture of the DCS-120 FLIM System

The DCS-120 system is a complete confocal laser scanning microscope for fluorescence lifetime imaging. The basic system is based on picosecond diode laser excitation, fast galvanometer-mirror scanning, confocal detection, and bh’s multi-dimensional TCSPC technique [1, 2, 3], see Fig. 1.

![Fig. 1: The DCS-120 scanner at a Zeiss Axio Observer (left) and at a Nikon 2000 TE microscope (middle). The TCSPC and control electronics is located in bh ‘Simple Tau’ system (right).](image)

The DCS-120 system is highly modular. It is available with inverted microscopes of Zeiss, Nikon, and Olympus. Moreover, the DCS-120 scanner can be adapted to conventional microscopes of almost any type and manufacturer, and be used with a variety of different lasers and detectors. DCS-120 systems can also be upgraded with tuneable excitation, or with multiphoton excitation and non-descanned detection. The general system architecture is shown in Fig. 2.

DCS-120 Scan Head

The DCS-120 scan head contains the complete beam deflection and confocal detection optics. The laser beams are deflected by fast-moving galvanometer mirrors, and sent down the microscope beam path. The axis of the galvanometer mirrors is projected into the plane of the microscope lens. With the motion of the galvanometer mirrors the laser focus thus scans over the focal plane in the
The scanning is controlled by a bh GVD-120 scan controller. The control of the scanner is fully integrated in the instrument software.

The emission light is collected through the microscope lens. The beam is descanned by the galvanometer mirrors, separated from the excitation beam, split into two channels of different wavelength or different polarisation and focused into pinholes in a plane conjugate with the focal plane in the sample. Out-of-focus light is thus suppressed. Please see [1] for details of the optical system.

The DCS-120 scan head comes in different versions. For use with two ps diode lasers it has a dual-band dichroic beamsplitter that matches the wavelengths of the lasers used. For use with tuneable lasers it is available with a wideband beamsplitter [12]. The wideband beamsplitter version is also recommended if the scanner is to be used with more than two diode lasers of different wavelengths.

**Fig. 2:** Basic system architecture of the DCS-120

**Picosecond Diode Lasers**

In the basic configuration, the DCS-120 system has one or two bh BDL-SMC picosecond diode lasers. The standard laser wavelengths are 405 nm, 445 nm, 473 nm, or 488 nm. Diode lasers with wavelengths of 640 nm, 685 nm and 785 nm are available on request. The diode lasers are coupled into the DCS-120 scan head via single-mode fibres.

**Femtosecond Titanium-Sapphire Lasers**

With a femtosecond titanium-sapphire laser the DCS-120 system can be converted into a multiphoton microscope [10]. In order to maintain femtosecond pulse width the Ti:Sa laser must be free-beam coupled into the DCS-120 scan head. To exploit the deep-tissue imaging capability of multiphoton excitation non-descanned detectors are available, see below.

**Tuneable Excitation**

To support full tuneability a ‘wideband’ (WB) version of the DCS-120 scanner is available [12]. Images obtained with a Toptica Ichrome [11] laser are shown in Fig. 12, page 7. The laser is coupled into the scanner via the same single mode fibres as the diode lasers.
Confocal Detectors

The detectors are directly coupled to optical ports at the back of the scanner. Coupling loss, reflections, or pulse dispersion in optical fibres are thus avoided. A number of different detectors is available. The standard detectors are the bh HPM-100-40 hybrid detector modules [2, 5]. HPM-100-50 GaAsP hybrid detectors, PMH-100 PMT modules, or ultra-fast Hamamatsu R3809U MCP PMTs can be used as well. For multi-spectral FLIM, the bh MW-FLIM multi wavelength detector can be attached to either of the DCS-120 output channels [17].

Non-descanned detectors

For multiphoton FLIM systems non-descanned detection is available. Adapters for the HPM-100 detectors, the PMC-100 detectors, the R3809U detectors or the MW-FLIM detector are available for the commonly used microscopes [13, 3].

TCSPC FLIM technique

The signals from the detectors are recorded by bh’s proprietary multi-dimensional TCSPC technique [2, 3]. In the standard configuration two bh SPC-150 TCSPC modules are used. Due to the dual-channel TCSPC architecture lifetime images can be recorded at unprecedented count rates and extremely short acquisition times [24].

The TCSPC and control electronics of the DCS-120 system comes as a compact ‘Simple-Tau’ system. The TCSPC cards, the scan controller, and the detector controller are contained in an electronics box that is connected to a laptop computer via a bus extension interface [3]. The Simple-Tau system of the DCS-120 is shown in Fig. 1, page 1.

The DCS-120 system allows the user to exploit the full range of functions of the bh TCSPC technique [3]. Single- and multi-exponential lifetime images [16], multi-spectral lifetime images [17], steady-state images, phosphorescence lifetime images [6, 7], fluorescence decay curves at single points [3], transient lifetime effects within a line scan [8, 9], and sequences of lifetime images [24] can be recorded as well as fluorescence correlation data, photon counting histograms, of BIFL data [22]. Please see [1] and [3] for details.

DCS-120 Features

Fast Beam Scanning

The DCS-120 uses fast beam scanning by galvanometer mirrors. A complete frame is scanned within a time from 100 ms to a few seconds, with pixel dwell times down to one microsecond.

Beam scanning is mandatory for live cell imaging in that it avoids induction of cell motion by exertion of dynamic forces to the sample. Moreover, live cell imaging requires a fast preview function for fluorescence images for sample positioning and focusing. This can only be provided if the beam is scanned at high frame rate.

Fig. 3: Bacteria in motion. Autofluorescence, acquisition speed 2 images per second, scan speed 6 frames per second
Fast scanning is also the basis of recording fast FLIM time series. Time-series recording can, of course, be only as fast the scanner is able to scan one frame. With the DCS-120 time-series can be recorded as fast as two images per second.

**Suppression of out-of-focus light by confocal detection**

The confocal detection principle efficiently suppresses out-of-focus light, please see [1]. It avoids loss in contrast by out-of-focus blur, and contamination of the recorded decay functions by decay components from other sample planes or from the embedding medium. An example is shown in Fig. 4. A non-confocal image (from a non-descanned detector) is shown on the left, a confocal image taken through a pinhole of 1 Airy unit on the right.

![Fig. 4: Non-confocal fluorescence lifetime image (left) in comparison to confocal image (right)](image)

**High-Efficiency GaAsP Hybrid Detectors**

The new bh HPM-100-40 GaAsP hybrid detectors of the DCS-120 combine SPAD-like sensitivity with the large active area of a PMT [2, 3, 5]. The large area avoids any alignment problems, and allows light to be efficiently collected even through large pinholes, see Fig. 5. In contrast to SPADs, there is no ‘diffusion tail’ in the temporal response. Moreover, the hybrid detectors are free of afterpulsing. The absence of afterpulsing results in improved contrast, higher dynamic range of the decay curves recorded, and in the capability to obtain FCS data from a single detector.

![Fig. 5: Fluorescence lifetime images recorded with an HPM-100-40 hybrid detector (left) and with an id-100-50 SPAD (right). Images and decay functions at selected cursor position.](image)

**Integrated Scanner Control**

The DCS-120 system is controlled by the bh SPCM TCSPC software. The control of the scanner is fully integrated, see Fig. 6. The scanner control panel allows the user to select the image format, scan rate, scan area, and to control the lasers. Changes in the scan parameters can be made at any time, even without stopping the scan.

The DCS-120 has automatic scan speed control. It automatically selects the fastest possible scan rate available for the scan parameters used.
**Fast Preview Function and Interactive Scanner Control**

The DCS-120 has a fast preview function that scans the sample at high speed, and displays fluorescence images in intervals of one second or less. With the preview function it is easy to bring the sample into focus, shift it in the desired position, and select the region to be scanned. The scanner control is fully integrated in the SPCM data acquisition software. The zoom factor and the position of the scan area can be adjusted via the scanner control panel or via the cursors of the display window. Changes in the scan parameters are executed online, without stopping the scan.

---

**FLIM Data Acquisition**

After the desired focal plane and scan area have been selected the preview is stopped and the acquisition of the FLIM data is started. During the acquisition the SPCM software displays intermediate results in predefined intervals, usually every few seconds. The acquisition can be stopped after a defined acquisition time or by a user command when the desired signal-to-noise ratio has been reached. The second way is to be preferred because the count rates of different samples may differ by at least an order of magnitude. The acquisition time required to reach a given lifetime accuracy may therefore vary in a wide range [1, 3].
Dual-Wavelength FLIM

With its two detection channels, the DCS-120 system records in two wavelength intervals simultaneously. The signals are detected by separate detectors and processed by separate TCSPC modules [1, 3]. There is no intensity or lifetime crosstalk due to counting loss or pile up. Even if one channel overloads the other channel is still able to produce correct data.

Laser Wavelength Multiplexing

The two diode lasers of the DCS-120 system can be multiplexed on a pixel-by-pixel, line-by-line, or frame-by-frame basis [1]. An example of a wavelength-multiplexed recording is shown in Fig. 10. Laser multiplexing helps discriminate the signals of several fluorophores, or allows one to excite two fluorophores that cannot efficiently be excited at the same wavelength. The capability of fast multiplexing avoids artefacts by photobleaching or dynamic effects in the sample.
**High-Resolution Images**

The pixel numbers of FLIM images can be increased up to 2048 x 2048. Fig. 11 shows an example. The useful pixel resolution is thus rather limited by the performance of the microscope lens than by the capabilities of the DCS-120 system.

**Tuneable Excitation**

The DCS-120 WB wideband version can be used with tuneable excitation. Images obtained with a Toptica Ichrome laser [16] are shown in Fig. 12.
Multiphoton Imaging Capability

With a femtosecond titanium-sapphire laser the DCS-120 system converts into a multiphoton microscope. Multiphoton excitation penetrates deep into biological tissue. Moreover, excitation occurs only in the focus of the laser. The fluorescence can therefore be detected through a large pinhole or by a non-descanned detector [13]. Fluorescence photons scattered on the way out of the sample are thus detected more efficiently than in a confocal system. The result is that clear images are obtained from deep tissue layers.

Fig. 13: Pig skin, autofluorescence, image in different depth in the sample. Amplitude-weighted lifetime of triple-exponential decay model. Excitation 805 nm, 512x512 pixels, 256 time channels. Zeiss Axio Observer Z1, Water C apochromate NA=1.2, non-descanned detection, HPM-100-40 hybrid detector.

Multi-Wavelength FLIM

The bh multispectral FLIM detector can be used to simultaneously record in 16 wavelength intervals [15, 2, 3, 17]. An example is shown in Fig. 14.

Fig. 14: Multi-wavelength FLIM. Human epithelium cells, autofluorescence. Excitation at 405 nm.

There is no time gating, no wavelength scanning and, consequently, no loss of photons by rejecting any part of the signal. The system thus reaches near-ideal recording efficiency. Moreover, dynamic effects in the sample or photobleaching do not cause distortions in the spectra or decay functions.

Z Stack Recording

In combination with the Zeiss Axio Observer microscope the DCS-120 system is able to record z-stacks of FLIM images [1]. The sample is continuously scanned. For each plane, a FLIM image is acquired for a specified ‘collection time’. Then the data are saved in a file, the microscope is commanded to step to the next plane, and the next image is acquired. The procedure continues for a specified number of Z planes. A Z stack of autofluorescence images taken at a water flee is shown in Fig. 15.
Fig. 15: Z stack recording, part of a water flea, autofluorescence. Images 256x256 pixels, 256 time channels. 15 steps in Z, step width 4 um.

**Time-Series FLIM**

Time-series FLIM is available for all system versions, and all detectors [1, 3]. With the SPC-152 dual-channel TCSPC systems time series as fast as 2 images per second can be obtained [24]. A time series taken at a moss leaf is shown in Fig. 16. The fluorescence lifetime of the chloroplasts changes due to the Kautski effect induced by the illumination.

Fig. 16: Time-series FLIM, 2 images per second. Chloroplasts in a leaf, the fluorescence lifetime of the chlorophyll decreases with the time of exposure.

**PLIM: Phosphorescence Lifetime Imaging**

The DCS-120 is able to simultaneously record fluorescence and phosphorescence lifetime images. The technique is based on modulating the ps diode laser synchronously with the pixel clock of the scanner. Fluorescence is recorded during the on time, phosphorescence during the off time of the laser. Please see [6, 7] or [3] for details.

Fig. 17: Phosphorescence lifetime imaging of inorganic luminophor. Left: Lifetime image. Right: Decay curve at selected position within the image.

**FLITS: Fluorescence Lifetime-Transient Scanning**

FLITS records transient effects in the fluorescence lifetime of a sample along a one-dimensional scan. The technique is based on building up a photon distribution over the distance along the scan,
the arrival times of the photons after the excitation pulses, and the experiment time after a stimulation of the sample. The maximum resolution at which lifetime changes can be recorded is given by the line scan time. With repetitive stimulation and triggered accumulation transient lifetime effects can be resolved at a resolution of about one millisecond [8, 9].

![Fluorescence lifetime images](image1)

**DCS-120 MACRO: Scanning Macroscopical Objects**

The DCS-120 MACRO version scans objects as large as 15 mm in the primary image plane of the scan head. An image obtained with the DCS-120 MACRO is shown in Fig. 19.

![Fluorescence lifetime images](image2)

**FCS**

The bh GaAsP hybrid detectors deliver highly efficient FCS. Because the detectors are free of afterpulsing there is no afterpulsing peak in autocorrelation data [2]. Thus, accurate diffusion times and molecule parameters are obtained from a single detector. Compared to cross-correlation of split signals, correlation of single-detector signals yields a four-fold increase in correlation efficiency. The result is a substantial improvement in the SNR of FCS recordings.
Fig. 20: FCS curve recorded by a single HPM-100 detector. There is no afterpulsing peak, and the efficiency is four times the efficiency of cross-correlation.

**Data Analysis**

Two data analysis packages are available, see Fig. 21. SPCImage runs a de-convolution on the decay data in the pixels of FLIM data. It uses single, double, or triple-exponential decay analysis to produce false-colour images of lifetimes, amplitudes, or intensities of decay components, or of ratios of these parameters [1, 14]. It displays single and double-exponential FRET data, and histograms of all the parameters calculated. SPCImage interacts directly with the SPCM data acquisition software.

The ‘Optispec’ package focuses on the analysis of multi-dimensional data, such as multi-spectral FLIM data or FLIM time series. It automatically analyses a large number of images or other data sets of similar origin. It uses single, double, or triple-exponential decay models. The decay parameters can either be independent for the individual data sets, or selected parameters can be fit globally.

Fig. 21: SPCImage (left) and Optispec data analysis (right)

DCS-120 data are compatible with multi-parameter FLIM analysis [21, 22, 28] and phasor analysis [21] in the frequency domain.

**Typical Applications**

The advantage of FLIM over other fluorescence imaging techniques is that the fluorescence lifetime of a fluorophore depends on its molecular environment but not on the concentration, see Fig. 22. If fluorescence in a sample is excited (Fig. 22, left) the emission intensity depends both on the concentration of the fluorophore and on possible interaction of the fluorophore with its molecular environment. Changes in the concentration, cannot be distinguished from changes in the molecular environment. Spectral measurements (second right) are able to distinguish between different fluorophores. However, changes in the local environment usually do not cause changes in the shape of the spectrum. The fluorescence lifetime of a fluorophore (Fig. 22, right), within reasonable limits,
does not depend on the concentration but systematically changes on interaction with the molecular environment.

Fig. 22: Fluorescence. Left to right: Excitation light is absorbed by a fluorophore, and fluorescence is emitted at a longer wavelength. The fluorescence intensity varies with concentration. The fluorescence spectrum is characteristic of the type of the fluorophore. The fluorescence decay function is an indicator of interaction of the fluorophore with its molecular environment.

By using the fluorescence lifetime, or, more precisely, the shape of the fluorescence decay function, molecular effects can therefore be investigated independently of the unknown and usually variable fluorophore concentration [3, 18, 25]. Common FLIM applications are ion concentration measurements, probing of protein interaction via FRET, and the probing of metabolic activity and cell viability via the fluorescence lifetimes of NADH and FAD. FLIM may also find application in plant physiology because the fluorescence lifetime of chlorophyll changes with the photosynthesis activity.

**Förster Resonance Energy Transfer: FRET**

A particularly efficient energy transfer process is Förster resonance energy transfer, or FRET. The effect was found by Theodor Förster in 1946 [23]. FRET is a dipole-dipole interaction of two molecules in which the emission band of one molecule overlaps the absorption band of the other. In this case the energy from the first molecule, the donor, transfers into the second one, the acceptor, see Fig. 23, left. FRET results in an extremely efficient quenching of the donor fluorescence and, consequently, in a considerable decrease of the donor lifetime, see Fig. 23, right.

The energy transfer rate from the donor to the acceptor increase with the sixth power of the reciprocal distance. Therefore it is noticeable only at distances shorter than 10 nm [25]. FRET is used as a tool to investigate protein-protein interaction. Different proteins are labelled with the donor and the acceptor, and FRET is used as an indicator of the binding between these proteins. Steady-state FRET measurements have the problem that the relative concentration of donor and acceptor varies, that the donor emission spectrally extends into the acceptor emission, and that a fraction of the acceptor is excited directly. FLIM does not have these problems because all it needs is to record a lifetime image at the donor emission wavelength. FRET is the most frequent FLIM application, please see [3] for references.

Fig. 24 shows FRET in a cultured live HEK cell. The cell is expressing two proteins, one labelled with CFP, the other with YFP. FRET occurs in the places where the proteins interact.
associated changes in the donor lifetime are clearly visible in the lifetime image shown in Fig. 24, left.

FLIM is not only able to detect FRET without interference by donor and acceptor bleedthrough, it is even delivers independent images of the donor-acceptor distance and the fraction of interacting donor. Such images can be obtained by double-exponential analysis of the FLIM data: The interacting donor fraction delivers a fast, the non-interacting fraction a slow decay component. The ratio of the two lifetimes is directly related to the donor-acceptor distance, the ratio of the amplitudes of the components is the ratio of interacting and non-interacting donor. Images which resolve these two parameters of the FRET system are shown in Fig. 24, middle and right.

Remarkably, double exponential FRET does not need an external lifetime reference: The reference lifetime is the slow decay component, originating from the non-interaction donor. Please see [1, 2, 3] for details and for further references.

![Fig. 24: FRET in HEK cell expressing proteins labelled with CFP and YFP. Left: Lifetime image at donor wavelength, showing lifetime changes by FRET. Middle and right: FRET results obtained by double-exponential lifetime analysis. Ratio of the lifetimes of the decay components, \( t_2/t_1 = \tau_0/\tau_{\text{fret}} \), and ratio of the interacting and non-interacting donor fractions, \( a_1/a_2 = N_{\text{fret}}/N_0 \).](image)

**Autofluorescence**

Biological tissue contains a wide variety of endogenous fluorophores [26]. However, the fluorescence spectra of endogenous fluorophores are broad, variable, and poorly defined. Moreover, absorbers present in the tissue may change the apparent fluorescence spectra. It is therefore difficult to disentangle the fluorescence components by their emission spectra alone. Autofluorescence lifetime detection is expected to add an additional separation parameter to the analysis of the data.

More important, the autofluorescence intensities and lifetimes contain information about the binding, the metabolic state and the microenvironment of the fluorophores. Especially interesting are the fluorescence signals from coenzymes, such as flavin adenine nucleotide (FAD) and nicotinamide adenine dinucleotide (NADH). It is known that the fluorescence lifetimes of NADH and FAD depend on the binding [25]. The lifetimes, the ratio of bound and unbound NADH, and the NADH / FAD intensity ratio also depend on the metabolic state [20], and on the redox state [19]. The NADH and FAD fluorescence intensities and lifetimes are therefore used to detect precancerous and cancerous alterations [27]. For an overview about the literature please see [3].

Fig. 25 shows an example of how autofluorescence signals change with the oxygen concentration. Yeast cells were kept in a sugar solution. They produce \( \text{CO}_2 \) which washes out the oxygen from the solution. The left image was recorded under such conditions. Only a few cells are visible Fig. 25, left and middle, the other ones are extremely dim. The image in Fig. 25, right, was recorded after the solution had been saturated with oxygen. The difference in the fluorescence behaviour is striking.
Fig. 25: Autofluorescence of yeast cells. Left and middle: Saturated with CO₂, different intensity scale of the same data set. Right: Saturated with O₂. Excitation 405 nm, detection at 540 nm.

Fig. 26 shows a pig skin autofluorescence image obtained at 405 nm excitation wavelength. Due to the absence of exogenous fluorophores the fluorescence intensity is low. Nevertheless, the FLIM data contain enough photons for double-exponential decay analysis. The image on the left shows the amplitude-weighted mean lifetime, $t_m$. The image in the middle shows the ratio of the intensities, $q_1/q_2$, contained in the fast and the slow decay component. Two typical decay curves are shown on the right.

Fig. 26: Pig skin sample excited at 405 nm, detection from 460 to 500 nm. Double-exponential fit. Left: Amplitude-weighted lifetime. Middle: Intensity ratio of fast and slow decay component. Right: Decay curves in two spots of the image.

In the wavelength interval recorded the emission can be expected to be dominated by NADH fluorescence. The lifetimes of bound and unbound NADH are different. The $q_1/q_2$ ratio can therefore be expected to represent the intensity ratio of bound and unbound NADH. It should be noted that accurate NADH analysis, of course, requires spectral unmixing of the NADH signal from contributions of other fluorophores [20]. Due to the variability of the autofluorescence spectra and lifetimes, fluorescence contribution from other fluorophores, and the presence of unknown absorbers the task is extremely complicated. The prospects of unmixing the signals improve considerably with the availability of excitation wavelength multiplexing (Fig. 10, page 7) or tuneable excitation (Fig. 12, page 7).

Plant Physiology

Two examples of FLIM of plant tissue are shown in Fig. 27 and Fig. 28. The fluorescence is dominated by the fluorescence of chlorophyll and the fluorescence of flavines. Multi-wavelength FLIM images of a moss leaf recorded with the bh multi-spectral FLIM detector are shown in Fig. 27.
The fluorescence of chlorophyll competes with the energy transfer into the photosynthesis channels. Thus, the fluorescence lifetime and its change on illumination is a sensitive indicator of the photosynthesis efficiency. The change in the fluorescence lifetime of the chloroplasts in a moss leaf on exposure to light can recorded by time-series FLIM, see Fig. 28.

Faster effects down to the millisecond time scale can be recorded by FLITS, as shown in Fig. 18, page 10.

Summary

The DCS-120 system is a cost-efficient alternative to upgrading a ‘big’ laser scanning microscope with FLIM. Due to full integration of FLIM recording and scanner control the DCS-120 may even be easier to use and more flexible in providing advanced FLIM functions, such as Z stack FLIM, time-series FLIM, or phosphorescence lifetime imaging. Applications of FLIM make use of the fact that the fluorescence lifetime depends on the molecular environment of the fluorophore molecules but not on their concentration. The most common application is protein-interaction measurement by FRET, where FLIM delivers information not accessibly by steady-state fluorescence imaging techniques.

References

DCS-120 FLIM System

24. V. Katsoulidou, A. Bergmann, W. Becker, How fast can TCSPC FLIM be made? Proc. SPIE 6771, 67710B-1 to 67710B-7