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www.boselec.com boselec@world.std.com

Time Correlated Single Photon Counting Systems

The Becker & Hickl SPC-series Module Family

PC Based Systems

intelligent measurement and control systems

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Boston Electronics Corporation, 91 Boylston St, Brookline MA 02445 USA * (800)3477-5445 or (617)566-3821 * fax (617731-0935 * tcspc@boselec.com

	SPC-630	SPC-730	SPC-830	SPC-134	Time Harp 200
Target Application	Standard lifetime experiments Single Molecule Detection Stopped Flow Correlation Experiments FCS Experiments	Standard lifetime experiments, Lifetime imaging, Confocal and two-photon scanning Microscopy Multi parameter experiments Stopped Flow	Standard lifetime experiments, Lifetime imaging, Confocal and two-photon scanning Microscopy Multi parameter experiments Stopped Flow Single Molecule Detection Correlation Experiments FCS Experiments	Optical tomography Single Molecule Stopped Flow Correlation Experiments FCS Experiments	Standard lifetime Single Molecule Microscope with scan stage Correlation Experiments FCS Experiments
No. of TCSPC Channels	1	1	1	4	1
Modules operable in parallel	4 x SPC-630	4 x SPC-730	4 x SPC-830	1 x SPC-134	
Conversion Principle	TAC - ADC with error reduction Patent DE 43 39 784 A1	TAC - ADC with error reduction Patent DE 43 39 784 A1	TAC - ADC with error reduction Patent DE 43 39 784 A1	TAC - ADC with error reduction Patent DE 43 39 784 A1	Time-to-Digital Converter
Detector Channel Sync Channel	Constant Fraction Constant Fraction	Constant Fraction Constant Fraction	Constant Fraction Constant Fraction	Constant Fraction Constant Fraction	Constant Fraction Level Trigger
Time Resolution	820 fs per time channel	820 fs per time channel	820 fs per time channel	820 fs per time channel	40 ps per time channel
Diff. nonlinearity	0.6% to 1% pp, <0.5% rms	0.6% to 1% pp, <0.5% rms	0.6% to 1% pp, <0.5% rms	0.6% to 1% pp, <0.5% rms	<6%pp, <0.5% rms
Detectable Lifetimes	2 ps to 2µs	2 ps to 2µs	2 ps to 2µs	2 ps to 2µs	<100ps to 4.5µs
Histogramming Process	Hardware, on board histogram memory	Hardware, 4-dimensional, on board histogram memory	Hardware, 4-dimensional, on board histogram memory	Hardware, on board histogram memory	Hardware, on board histogram memory
Image size for fast scan modes		max. 256 x 256 pixels	max 4096 x 4096 pixels		
Dead Time	125 ns	180 ns	125 ns	125 ns	<350 ns
Useful continuous count rate, Histogram Modes, 50% loss, per module	4 MHz	2.8 MHz	4 MHz	16 MHz (overall for 4 channels)	1.4 MHz
Peak Count Rate, histogram modes, 50% loss, per moduel	4 MHZ	2.8 MHz	4 MHz	16 MHz (overall for 4 channels)	1.4 MHz
Continuous count rate, time-tag modes	0.4...0.8 MHz, depends on computer speed and background activity		3...4 MHz, depends on computer speed and background activity	0.4...0.8 MHz, depends on computer speed and background activity	Depends on computer speed and background activity
Peak count rate, time-tag modes, 50% loss	4 MHz independent of computer speed		4 MHz independent of computer speed	16 MHz independent of computer speed	Depends on computer speed and background activity
on-board FIFO buffer size, time tag modes	128,000 photons or 256,000 photons		8 Million photons	512,000 photons	128,000 photons
Macro time resolution in time tag (FIFO) modes	50 ns		50 ns from internal clock or 12ns to 100 ns from sync (laser)	50 ns from internal clock or 12ns to 100 ns from sync (laser)	100ns
Scan rate, Scan syn in mode		down to 100ns per pixel independent of computer speed	down to 100ns per pixel independent of computer speed		
Multi-Detector Operation	yes Patent DE 43 39 787 A1	yes Patent DE 43 39 787 A1	yes Patent DE 43 39 787 A1	yes Patent DE 43 39 787 A1	yes
No of curves in memory	2 x 64 to 4096	1024 to 65,536	4096 to 2,000,0000	2 x 32 to 2 x 2048 per TCSPC channel	2 x 32
Min. time per histogram	1µs in continuous flow mode	100ns in scan sync in/out mode	100ns in scan sync in/out mode	1µs in continuous flow mode	1µs in ext sync mode

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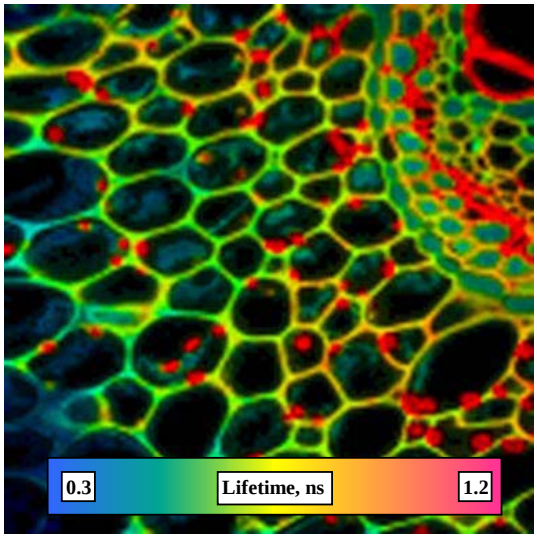
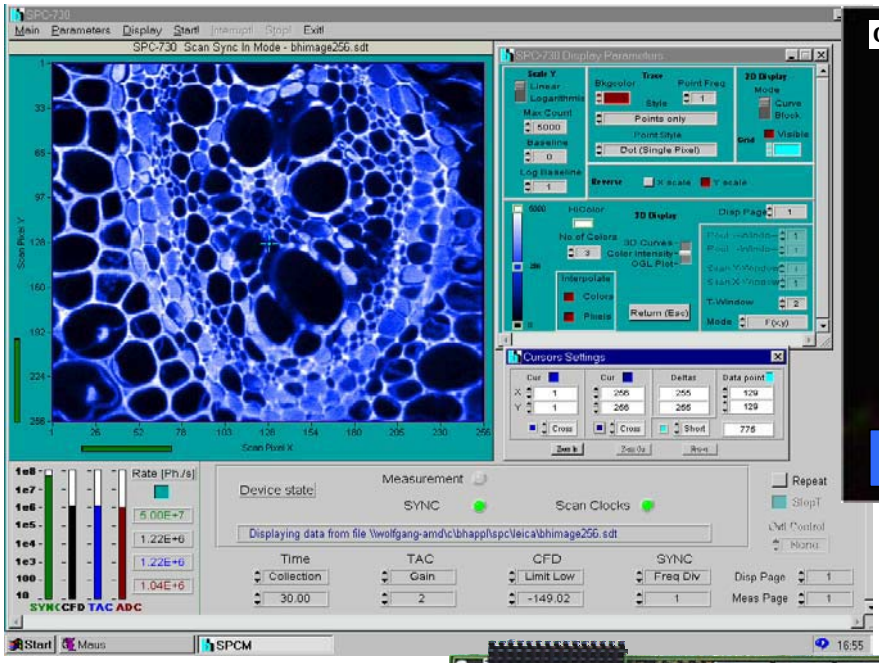
	SPC-630	**SPC-730**	**SPC-830**	**SPC-134**	**Time Harp 200**
Available multi-detector extension devices for	4 MCPs, 4 PMTs 8 MCPs, 8 PMTs 8 APDs 16 channel pmt head	4 MCPs, 4 PMTs 8 MCPs, 8 PMTs 8 APDs 16 channel pmt head	4 MCPs, 4 PMTs 8 MCPs, 8 PMTs 8 APDs 16 channel pmt head	4 MCPs, 4 PMTs 8 MCPs, 8 PMTs 8 APDs	4 APDs
Operating Modes	Single Oscilloscope 2 dimensional f(xyt) Sequence f(t,T), fi(t,ext) Spectrum fi(T), fi(ext) Continuous Flow (unlimited seq.) Time Tag (FIFO)	Single Oscilloscope 2 dimensional f(xyt) Sequence f(t,T), fi(t,ext) Spectrum fi(T), fi(ext) Imaging (Sync In, Sync Out, XY in, XY out)	Single Oscilloscope 2 dimensional f(xyt) Sequence f(t,T), fi(t,ext) Spectrum fi(T), fi(ext) Imaging (Sync In, Sync Out, XY in) Time Tag (FIFO)	Single Oscilloscope 2 dimensional f(xyt) Sequence f(t,T), fi(t,ext) Spectrum fi(T), fi(ext) Continuous Flow (unlimited seq.) Time Tag (FIFO)	Integration Oscilloscope Sequence f(t,T) Contiuous Time-tag (Option)
Experiment Trigger	Start of measurement Start of sequence Each step of sequence	Start of measurement Start of sequence Each step of sequence Frame Clock, Line Clock, Pxl Clock	Start of measurement Start of sequence Each step of sequence Frame Clock, Line Clock, Pxl Clock	Start of measurement Start of sequence Each step of sequence	Start of measurement Start of sequence
Triggered accumulation of sequences	yes	yes		yes	yes
Detector / Experiment control (Own products only)	Preamplifiers with detector overload protection, PMH-100 Detector modules, PML-16 multichannel detector head, DCC-100 Detector Controller , STP-340 Step Motor Controller, Routers for MCPs, PMTs, APDs, Dual ADC module for XY In operation	Preamplifiers with detector overload protection, PMH-100 Detector modules, PML-16 multichannel detector head, DCC-100 Detector Controller , STP-340 Step Motor Controller, Routers for MCPs, PMTs, APDs, Dual ADC module for XY In operation, Adapters for Zeiss, Leica, Olympus and Biorad laser scanning microscopes	Preamplifiers with detector overload protection, PMH-100 Detector modules, PML-16 multichannel detector head, DCC-100 Detector Controller , STP-340 Step Motor Controller, Routers for MCPs, PMTs, APDs, Dual ADC module for XY In operation, Adapters for Zeiss, Leica, Olympus and Biorad laser scanning microscopes	Preamplifiers with detector overload protection, PMH-100 Detector modules, PML-16 multichannel detector head, DCC-100 Detector Controller , STP-340 Step Motor Controller, Routers for MCPs, PMTs, APDs	Preamplifiers with detector overload protection, Routers for APDs
Free Documentaion available on web site	SPC Manual, 165 pages; TCSPC Introduction, 5 pages; Upgrading laser scanning microscopes for lifetime imaging; Controlling SPC modules; Protecting Photomultipliers; FRET measurements by TCSPC lifetime microscopy; Multi-wavelengthe TCSPC lifetime imaging; High count rate multichannel TCSPC for optical tomography; Optical Tomography: TCSPC Imaging of Female Breast; Setting up High Gain Detector Electronics for TCSPC Applications; Testing SPC Modules; 16 Channel Detector Head for TCSPC Modules; Routing Modules for Time-Correlated Single Photon Counting; Detector Control Module DCC100 Manual; **TCSPC Software is available and FREE**; Manual: Multi - SPC 32 bit Dynamic Link Library				Measurement examples
Related Products (Own products only)	SPC-300, SPC-330 TCSPC; SPC-400, SPC-430 TCSPC; SPC-500, SPC-530 TCSPC; MSA-100 1ns multiscaler; MSA-300 5ns multisclaer; PMS-400 and PMM-328 Gated photon counters / multiscalers; Picosecond Diode Lasers				Time Harp 100 Picosecond Diode Lasers

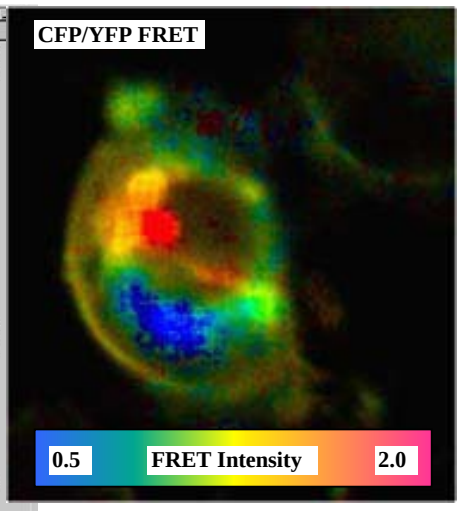
The TCSPC Microscopy Solution SPC-830

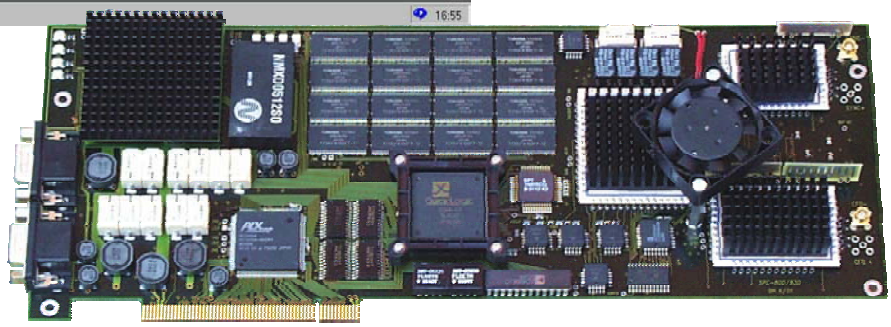
High Resolution Time-Correlated Single Photon Counting Imaging and FCS Module for Laser Scanning Microscopes

- Complete picosecond imaging system on single PC board
- Picosecond resolution
- Ultra-high sensitivity
- Multi detector capability
- High-speed on-board data acquisition
- Works at any scanning speed of microscope
- High resolution picosecond lifetime imaging
- FRET imaging
- High-resolution steady state imaging
- Single-point time-lapse lifetime analysis
- Single-point FCS / lifetime data
- Time channel width down to 813 fs
- Image size up to 4096 x 4096 pixels
- Electrical time resolution down to 8 ps fwhm / 4 ps rms
- Reversed start/stop: Laser repetition rates up to 200 MHz
- Useful count rate up to 4 MHz - dead time 125 ns
- Active and passive scanning control
- Software versions for windows 95 / 98 / 2000 / NT









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Covered by patents DE 43 39 784 A1 and DE 43 39 787

The TCSPC Microscopy Solution SPC-830

Photon Channel

Principle	Constant Fraction Discriminator
Time Resolution (FWHM / RMS, electr.)	7 ps / 4 ps
Opt. Input Voltage Range	- 50 mV to - 1 V
Min. Input Pulse Width	400 ps
Lower Threshold	- 20 mV to - 500 mV
Zero Cross Adjust	- 100 mV to + 100 mV

Synchronisation Channel

Principle	Constant Fraction Discriminator
Opt. Input Voltage Range	- 50 mV to - 1 V
Min. Input Pulse Width	400 ps
Threshold	- 20 mV to -500 mV
Frequency Range	0 to 200 MHz
Frequency Divider	1-2-4-8-16
Zero Cross Adjust	-100 mV to + 100 mV

Time-to-Amplitude Converter / ADC

Principle	Ramp Generator / Biased Amplifier
TAC Range	50 ns to 2 us
Biased Amplifier Gain	1 to 15
Biased Amplifier Offset	0 to 100% of TAC Range
Time Range incl. Biased Amplifier	3.3 ns to 2 us
min. Time / Channel	813 fs
TAC Window Discriminator	Any Window inside TAC Range
ADC Principle	50 ns 12 bit Flash ADC with Error Correction
Diff. Nonlinearity (dith width 1/8, 90% of TAC range)	< 0.5% rms, typically <1% peak-peak

Data Acquisition, Histogram Modes

Method	on-board 4-dimensional histogramming process over t, x, y, and detector channel number						
Dead Time	125ns, independent of computer speed						
Saturated Count Rate / Useful Count Rate	8 MHz / 4 MHz						
Number of Time Channels / Pixel	1	4	16	64	256	1024	4096
IImage Resolution (pixels), 1 Detector Channel	4096 x 4096	2048 x 2048	1024 x 1024	512 x 512	256 x 256	128 x 128	64 x 64
IImage Resolution (pixels), 4 Detector Channels	2048 x 2048	1024 x 1024	512 x 512	256 x 256	128 x 128	64 x 64	32 x 32
IImage Resolution (pixels), 16 Detector Channels	1024 x 1024	512 x 512	256 x 256	128 x 128	64 x 64	32 x 32	16 x 16
Counts / Time Channel	2^{16}-1						
Counts / Time Channel ('Single' mode, repeat and acquire)	2^{32}-1						
Overflow Control	none / stop / repeat and acquire						
Collection Time (per curve or per pixel)	100 ns to 1000 s						
Display Interval Time	10ms to 1000 s						
Repeat Time	0.1 ms to 1000 s						
Curve Control (Internal Routing / Scan Sync In Mode)	up to 262,144 decay curves						
Routing Control / Detector Channels	14 bit TTL / 16384						
Count Enable Control	1 bit TTL						
Control Signal Latch Delay	0 to 255 ns						
Experiment Trigger	TTL						

Data Acquisition, FIFO/BIFL Modes

Method	Time-tagging of individual photons and continuous writing to disk
Macro Time Resolution	50 ns
ADC Resolution / No. of Time Channels	12 bit / 4096
Dead Time	150 ns
Output Data Format (ADC / Macrotime / Routing)	12 / 12 /4
FIFO buffer Capacity (photons)	8 million photons

Multi Module Systems

Number of modules operable parallel	4

Operation Environment

Computer System	PC Pentium
Bus Connector	PCI
Power Consumption	approx. 20 W at +5V, 0.7 W at +12V
Dimensions	312 mm x 122 mm x 28 mm

Related Products and Accessories

Detector Heads (MCPs, PMTs), Multichannel Detector Heads, Routing Devices for Multichannel Measurements, Step Motor Controllers, Preamplifiers, PIN and Avalanche Photodiode Modules, ps Diode Lasers, Adapter Cables for Scanning Microscopes. SPC-600/630 TCSPC modules for single molecule and correlation spectroscopy, SPC-700/730 for imaging and SPC-134 for optical tomography. Please downlaod or call for individual data sheets. To control detectors and shutters please see DCC-100 detector controller.

Please visit our web site to download the manual, the device software and application notes.

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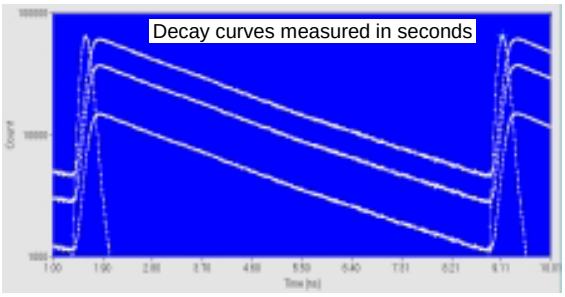
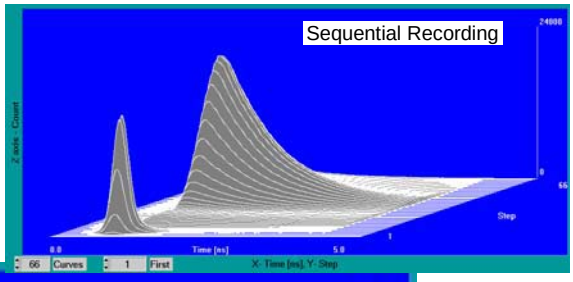
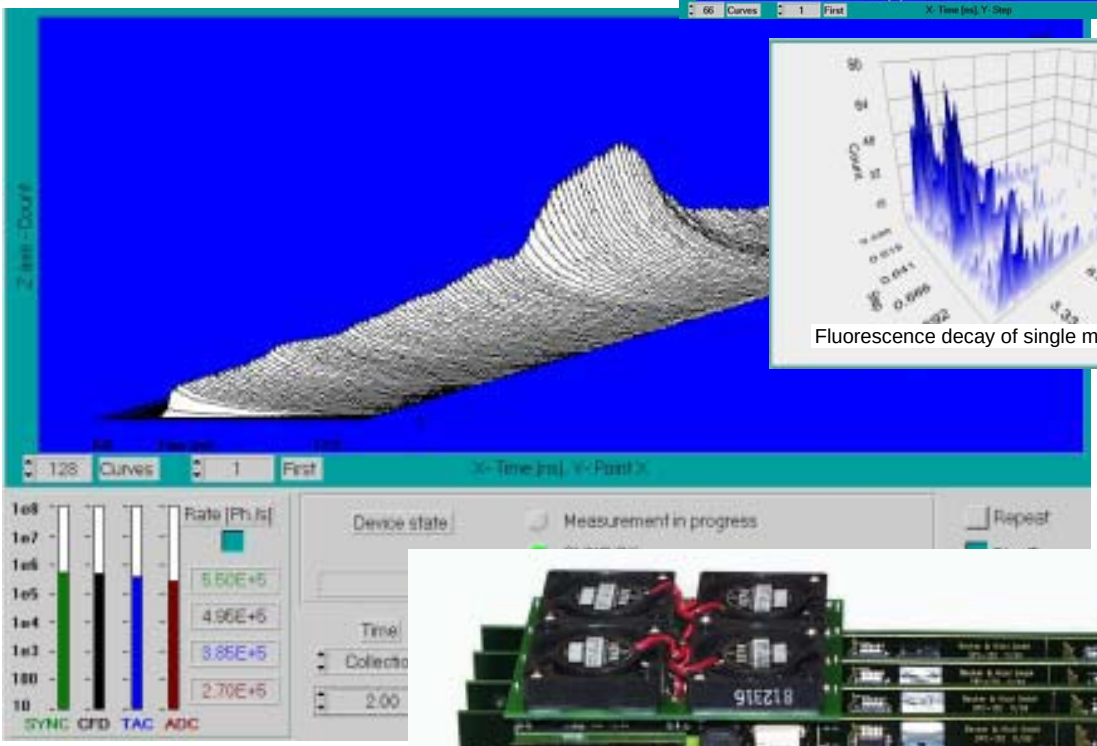
The TCSPC Power Package SPC-134

Four Channel Time-Correlated Single Photon Counting Module

- Four Completely Parallel TCSPC Channels
- Ultra-High Data Throughput
- Overall Count Rate 32 MHz
- Channel Count Rate 8 MHz (Dead Time 125ns)
- Dual Memory Architecture: Readout during Measurement
- Reversed Start/Stop: Repetition Rates up to 200 MHz
- Electrical Time Resolution down to 8 ps FWHM / 5 ps rms
- Channel Resolution down to 813 fs
- Up to 4096 Time Channels / Curve
- Measurement Times down to 0.1 ms
- Software Versions for Windows 95 / 98 / NT
- Direct Interfacing to most Detector Types
- Single Decay Curve Mode
- Oscilloscope Mode
- Seqential Recording Mode
- Spectrum Scan Mode with 8 Independent Time Windows
- Continuous Flow Mode for Single Molecule Detection







intelligent measurement and control systems

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SPC-134

Photon Channels

Principle	Constant Fraction Discriminator (CFD)
Time Resolution (FWHM / RMS, electr.)	8 ps / 5 ps
Opt. Input Voltage Range	- 50 mV to - 1 V
Lower Threshold	- 20 mV to - 500 mV
Upper Threshold	-
Zero Cross Adjust	- 100 mV to + 100 mV

Synchronisation Channels

Principle	Constant Fraction Discriminator (CFD)
Opt. Input Voltage Range	- 50 mV to - 1 V
Threshold	- 20 mV to -500 mV
Frequency Range	0 to 200 MHz
Frequency Divider	1-2-4
Zero Cross Adjust	-100 mV to + 100 mV

Time-to-Amplitude Converters / ADCs

Principle	Ramp Generator / Biased Amplifier
TAC Range	50 ns to 2 us
Biased Amplifier Gain	1 to 15
Biased Amplifier Offset	0 to 100% of TAC Range
Time Range incl. Biased Amplifier	3.3 ns to 2 us
min. Time / Channel	813 fs
ADC Principle	50 ns Flash ADC with Error Correction
Diff. Nonlinearity	< 2% rms

Data Acquisition

Dead Time	125ns			
max. Number of Curves in Memory	4096	1024	256	64
Number of Time Channels / Curve	64	256	1024	4096
max. Counts / Channel	2^{16}-1			
Overflow Control	none / stop / repeat and correct			
Collection Time	0.1 ms to 10000 s			
Display Interval Time	10ms to 1000 s			
Repeat Time	0.1 ms to 1000 s			
Curve Control (internal)	Programmable Hardware Sequencer			
Count Enable Control	1 bit TTL			
Measurement Trigger	TTL			

Operation Environment

Computer System	PC Pentium
Bus Connectors	PCI
Used PCI Slots	4
Power Consumption	approx. 18 W at +5V, 0.7 W at +12V
Dimensions	225 mm x 125 mm x 85 mm

Accessories and Associated Products

Detectors (MCPs, PMTs), Multichannel Detector Heads, Step Motor Controllers, Preamplifiers, PIN and Avalanche Photodiode Modules, ps Diode Lasers. Also available: SPC-3x0/4x0/500/5x0/6x0/7x0 time-correlated single photon counting modules, gated photon counters and multiscalers. Please call for individual data sheets and descriptions.

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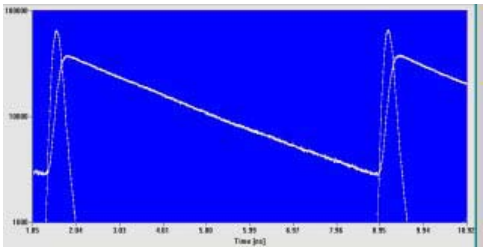
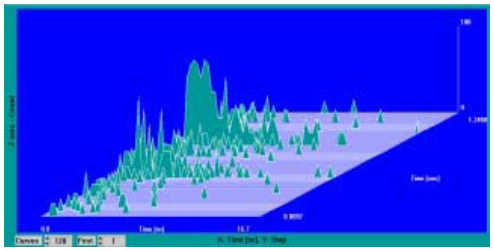
The TCSPC General Solution SPC-600/630

Time-Correlated Single Photon Counting Modules with dual Memory and PCI Bus

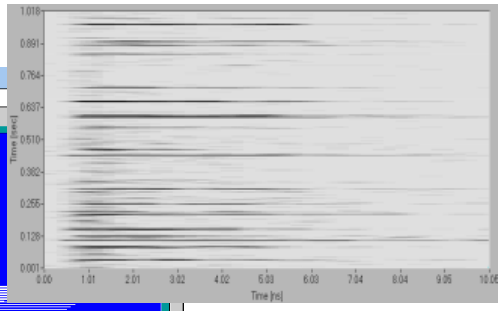
- Complete TCSPC Systems on single PC Boards
- Dual Memory Architecture: Unlimited Sequential Curve Recording
- Histogram Mode: Recording of Decay Curves
- FIFO Memory Mode: Continuous Recording by BIFL Method
- Reversed Start/Stop: Repetition Rates up to 200 MHz
- Electrical Time Resolution down to 8 ps FWHM / 5 ps rms
- Channel Resolution down to 813 fs
- Up to 4096 Time Channels / Curve
- Ultra High Count Rate: Up to 8 MHz (125 ns Dead Time)
- Measurement Times down to 0.1 ms
- Multi Detector Capability: Up to 128 Detector Channels
- Software Versions for Windows 3.1 / 95 / 98 / NT
- Optional Step Motor Controller for Wavelength or Sample Scanning
- Direct Interfacing to most Detector Types
- Single Decay Curve Mode
- Oscilloscope Mode
- Multiple Decay Curve Mode (Wavelength, Time or User Defined)
- Spectrum Scan Mode with 8 Independent Time Windows
- Multichannel X-Y-t-Mode
- Continuous Flow and BIFL Mode for Single Molecule Detection



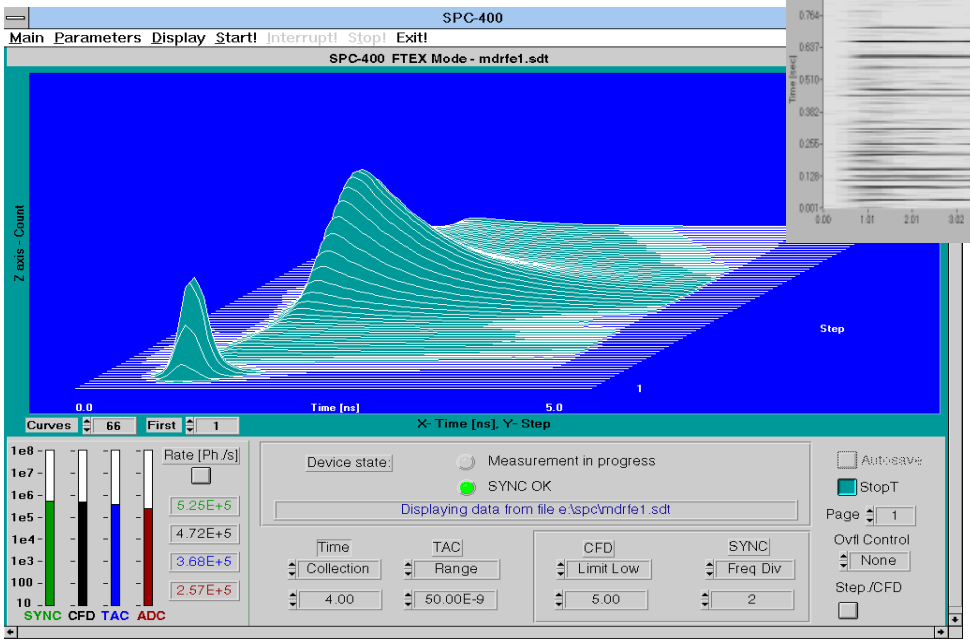
'Single' Mode: Decay curves measured within seconds

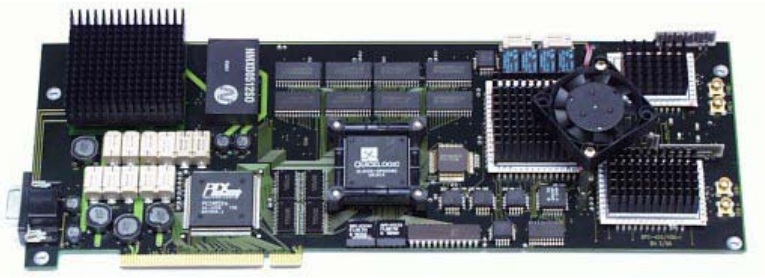


'Continuous Flow' Mode: Fluorescence decay from single molecules



'BIFL' Mode: Traces of single molecules





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SPC-600 SPC-630

Photon Channel	**SPC-600**	**SPC-630**
Principle	Constant Fraction Discriminator	
Time Resolution (FWHM / RMS, electr.)	13 ps / 7 ps	8 ps / 5 ps
Opt. Input Voltage Range	± 10 mV to ± 80 mV	- 50 mV to - 1 V
Lower Threshold	5 mV to 80 mV	- 20 mV to - 500 mV
Upper Threshold	5 mV to 80 mV	-
Zero Cross Adjust	-10 mV to + 10 mV	- 100 mV to + 100 mV

Synchronisation Channel	**SPC-600**	**SPC-630**
Principle	Constant Fraction Discriminator	
Opt. Input Voltage Range	± 10 mV to ± 50 mV	- 50 mV to - 1 V
Threshold	-	- 20 mV to -500 mV
Frequency Range	0 to 200 MHz	
Frequency Divider	1-2-4-8-16	
Zero Cross Adjust	-10 mV to + 10 mV	-100 mV to + 100 mV

Time-to-Amplitude Converter / ADC	
Principle	Ramp Generator / Biased Amplifier
TAC Range	50 ns to 2 us
Biased Amplifier Gain	1 to 15
Biased Amplifier Offset	0 to 100% of TAC Range
Time Range incl. Biased Amplifier	3.3 ns to 2 us
min. Time / Channel	813 fs
TAC Window Discriminator	Any Window inside TAC Range
ADC Principle	50 ns Flash ADC with Error Correction
Diff. Nonlinearity (dith. width 1/8)	< 0.5 % rms

Data Acquisition (Histogram Mode)				
Dead Time	125ns			
max. Number of Curves in Memory	4096	1024	256	64
max. Number of Detector Channels	128	128	128	32
Number of Time Channels / Curve	64	256	1024	4096
max. Counts / Channel	2^{16}-1			
Overflow Control	none / stop / repeat and correct			
Collection Time	0.1 us to 10000 s			
Display Interval Time	10ms to 1000 s			
Repeat Time	0.1 ms to 1000 s			
Curve Control (internal)	Programmable Hardware Sequencer			
Curve Control (external Routing)	7 bit TTL			
Add/Sub (Lock-in) Control	1 bit TTL			
Count Enable Control	1 bit TTL			
Control Signal Latch Delay	0 to 255 ns			

Data Acquisition (FIFO / BIFL Mode)		
ADC Resolution	12 bit	8 bit
Dead Time	150 ns	125 ns
Output Data Format (ADC / Macrotime / Routing)	12 / 24/ 8	8 / 17 / 3
FIFO buffer Capacity (photons)	128 k	256 k
Macro Timer Resolution	1 us, 24 bit	50ns, 17 bit
Curve Control (external Routing)	8 bit TTL	3 bit TTL
Count Enable Control	1 bit TTL	
Routing Signal Latch Delay	0 to 255 ns	

Operation Environment	
Computer System	PC Pentium or 486
Bus Connector	PCI
Power Consumption	approx. 20 W at +5V, 0.7 W at +12V
Dimensions	312 mm x 122 mm x 28 mm

Accessories and Associated Products

Detectors (MCPs, PMTs), Multichannel Detector Heads, Routing Devices for Multichannel Measurements, Step Motor Controllers, Preamplifiers, PIN and Avalanche Photodiode Modules, ps Diode Lasers. Also available: SPC-300/330/400/430/500/530 time-correlated single photon counting modules, gated photon counters and multiscalers. Please call for individual data sheets and descriptions.

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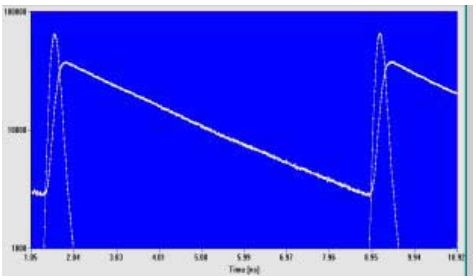
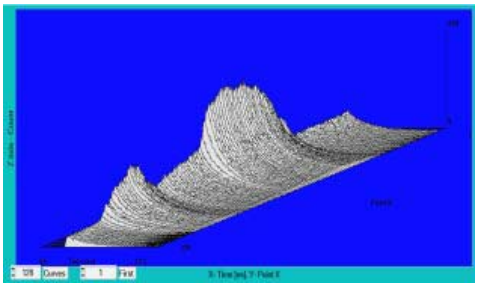
control systems

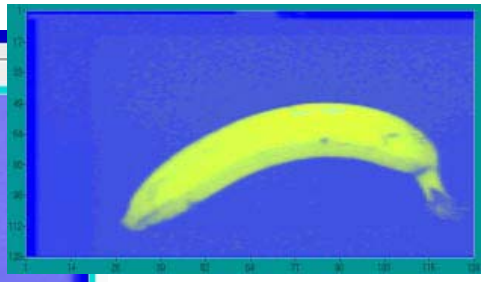
The ps Imaging Solution SPC-700/730

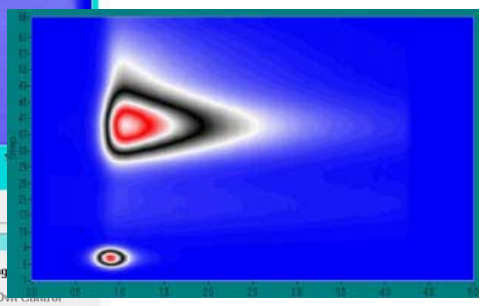
Time-Correlated Single Photon Counting Imaging Modules with Scanning Control and PCI Bus

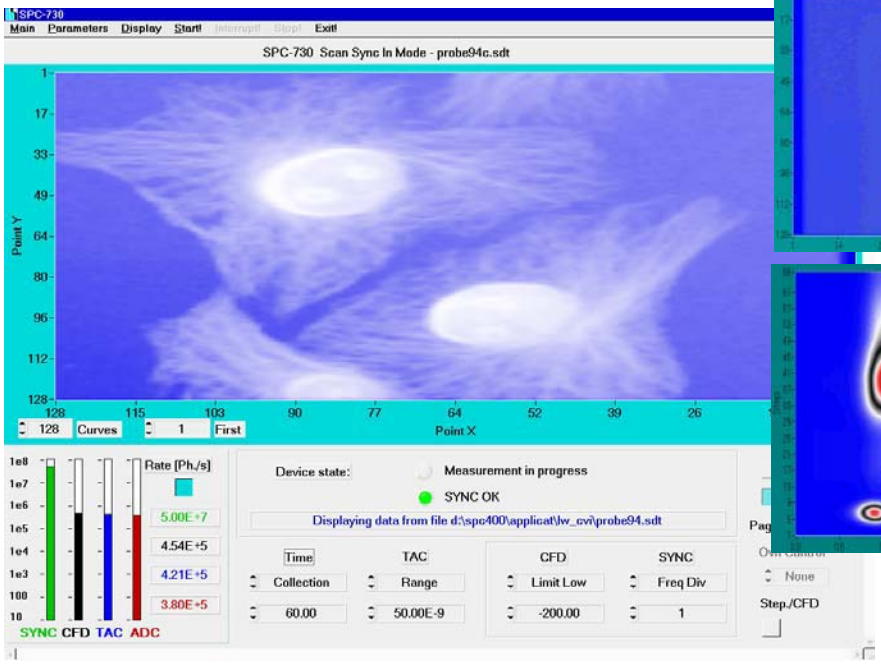


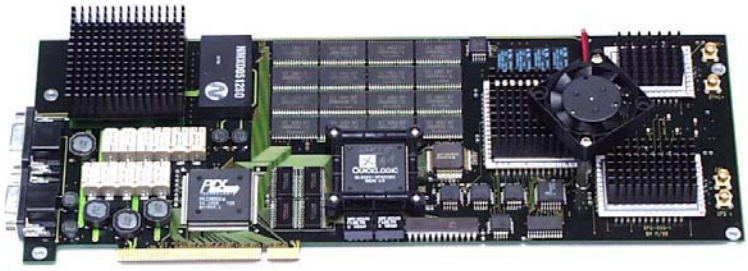


- Complete TCSPC Imaging Systems on single PC Boards
- Up to 65636 (256 x 256) Decay Curves in Memory
- Up to 4096 Time Channels / Curve
- Multi Detector Capability: Up to 128 x 128 Detector Channels
- Electrical Time Resolution down to 8 ps fwhm / 4 ps rms
- Channel Resolution down to 813 fs
- Reversed Start/Stop: Repetition Rates up to 200 MHz
- Count Rate up to 3 MHz
- Measurement Times down to 1 ms
- Active or Passive Scanning Control
- Optional Step Motor Controller
- Software Versions for Windows 3.1 / 95 / 98 / NT
- Single Decay Curve Mode
- Multiple Decay Curve Mode (Wavelength, Time or User Defined)
- Spectrum Scan Mode with 8 Independent Time Windows
- Oscilloscope Mode
- Multichannel X-Y-t-Mode
- Fast Object Scanning / Lifetime Imaging Modes









intelligent measurement and control systems

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SPC-700 SPC-730

Photon Channel	SPC-700	SPC-730
Principle	Constant Fraction Discriminator	
Time Resolution (FWHM / RMS, electr.)	13 ps / 7 ps	7 ps / 4 ps
Opt. Input Voltage Range	± 10 mV to ± 80 mV	- 50 mV to - 1 V
Lower Threshold	5 mV to 80 mV	- 20 mV to - 500 mV
Upper Threshold	5 mV to 80 mV	-
Zero Cross Adjust	-10 mV to + 10 mV	- 100 mV to + 100 mV

Synchronisation Channel	SPC-700	SPC-730
Principle	Constant Fraction Discriminator	
Opt. Input Voltage Range	± 10 mV to ± 50 mV	- 50 mV to - 1 V
Threshold	-	- 20 mV to -500 mV
Frequency Range	0 to 200 MHz	
Frequency Divider	1-2-4-8-16	
Zero Cross Adjust	-10 mV to + 10 mV	-100 mV to + 100 mV

Time-to-Amplitude Converter / ADC

Principle	Ramp Generator / Biased Amplifier
TAC Range	50 ns to 2 us
Biased Amplifier Gain	1 to 15
Biased Amplifier Offset	0 to 100% of TAC Range
Time Range incl. Biased Amplifier	3.3 ns to 2 us
min. Time / Channel	813 fs
TAC Window Discriminator	Any Window inside TAC Range
ADC Principle	50 ns Flash ADC with Error Correction
Diff. Nonlinearity (dith width 1/8, 90% of TAC range)	< 0.5% rms

Data Acquisition

Dead Time (from stop to next photon)	180 ns			
Number of Time Channels / Curve	64	256	1024	4096
max. Number of Curves in Memory	65536	16384	4096	1024
max. Square Scanning Area (pixels)	256 x 256	128 x 128	64 x 64	32 x 32
max. Number of Detector Channels	16384	16384	4096	1024
Counts / Channel	2^{16}-1			
Counts / Channel ('Single' mode, repeat and acquire)	2^{32}-1			
Overflow Control	none / stop / repeat and acquire			
Collection Time (per curve or per pixel)	100 ns to 1000 s			
Display Interval Time	10ms to 1000 s			
Repeat Time	0.1 ms to 1000 s			
Curve Control (Internal Routing)	up to 65536 Curves			
Curve Control (Passive Scanning)	14 bit TTL or SYNC Pulses from Scanner to SPC			
Scanning Control (Active Routing)	14 bit TTL or Sync Pulses from SPC to Scanner			
Count Enable Control	1 bit TTL			
Control Signal Latch Delay	0 to 255 ns			

Operation Environment

Computer System	PC Pentium or 486
Bus Connector	PCI
Power Consumption	approx. 20 W at +5V, 0.7 W at +12V
Dimensions	312 mm x 122 mm x 28 mm

Accessories

Detector Heads (MCPs, PMTs), Multichannel Detector Heads, Routing Devices for Multichannel Measurements, Step Motor Controllers, Preamplifiers, PIN and Avalanche Photodiode Modules, ps Diode Lasers, Adapter Cables for Scanning Microscopes. Please call for individual data sheets.

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intelligent
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High Performance
Photon Counting

August 2002

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SPCImage - Version 2.3

Data Analysis Software for Fluorescence Lifetime Imaging Microscopy

- Calculation of colored lifetime images with combined decay and intensity information
- Processing of images with up to 1M (1024 x 1024) decay curves and up to 256 time channels
- Multiexponential fit with deconvolution from instrumental response function
- Adaptive suppression of scattered light and dark noise
- Presentation of fit quality by weighted residuals and reduced χ^2 for any selected pixel
- Selectable region of interest (ROI) , display of parameter distribution within ROI
- Color and brightness postprocessing of intensity and lifetime images
- Dynamic data exchange (DDE) from SPC-730/830 measurement software or import of data files
- Export function of windows bitmap or TIFF-files with 24-bit color depth
- Windows 95/98™ and Windows NT 4.0/2000/XP™ compatible

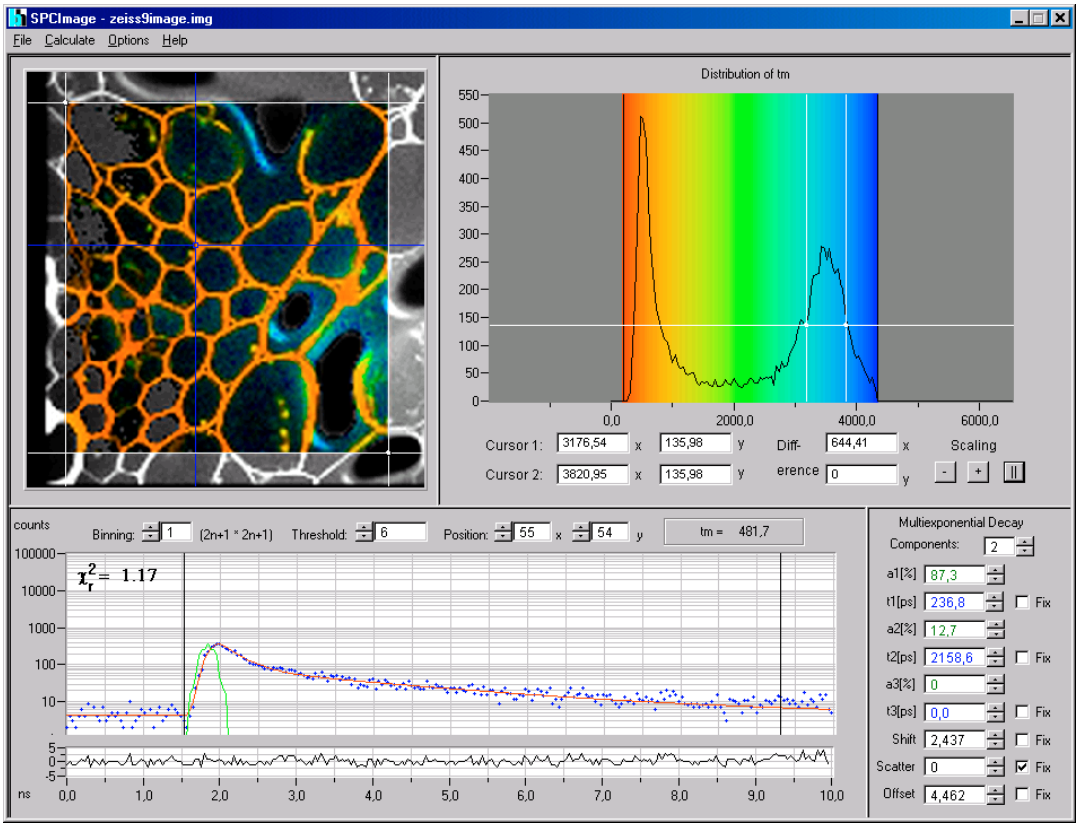


Fig. 1 : Graphical User Interface of the SPCImage software

1. Introduction

Parallel processing of the fluorescence intensity *and* the fluorescence lifetime will enhance performance of imaging systems significantly. Especially in fluorescence lifetime imaging microscopy (FLIM) it is an important benefit to exploit the dependency of fluorescence lifetime to physical parameters. In contrast to the intensity the lifetime is measured as an absolute value and therefore overcomes many problems which result from complicated calibration procedures.

The data analysis is of crucial relevance for gathering the desired information from time- and spatial-resolved fluorescence measurements. In principle two problems have to be solved a) derivation of a model function that is suitable for the description of time- and spatial-resolved fluorescence measurements and b) development of a fast algorithm for numerically fitting the model parameters to the individual data sets.

In the SPCImage software a deconvolution technique is used to separate the decays associated with the different pixels into the contributions which originate from different emitting species. Given a 2 dimensional set of fluorescence decay curves the model condenses the data by fitting it to a single exponential decay function with constant offset and a scattering correction. The model`s final values come from the underlying theory that the data can be approximated by a convolution of this 'pure decay' and a numerically recorded response function of the measuring system.

According to theory least-square fitting is a maximum likelihood estimation of the fitted parameters if the measurement errors are independent and normally distributed with constant standard deviation. Fluorescence measurements performed by time correlated single photon counting can fulfill the first requirement almost ideal since the data is collected from a large set of excitation pulses. However, the standard deviation of the time channels are not constant but obey a poisson statistic with a standard deviation of $\sigma^2 = N$, being N the number of the counts per channel. When relaxing the assumption of a constant standard deviation it is possible to work out a maximum likelihood estimation that holds for all cases where the number of counts per channel is not too small. In cases down to 4 counts the poisson distribution is still reasonably good approximated by a gauss function with $\sigma^2 = N$. Therefore the algorithm uses a "smart binning" technology which combines adjacent channels to achieve the above requirement for all channels with <4 counts. The result is a "weighted least square fitting for possion distributed data".

Main reason for this approximation is the possibility to deploy a very efficient algortihm to find the minimum of the weighted chi-square. This method is based on the Levenberg and Marquardt search algorithm [1], which depends on partial derivatives of the χ^2-value according to the fitting parameters. A time-optimized procedure is used to determine the derivatives of the χ^2in each pixel channel according to the parameters. It permits the simultaneous calculation of the convolution integral with the apparatus function.

There are important issues that go beyond the mere finding of best-fit parameters. Data are generally not exact. They are subject to measurement errors (called noise in the context of signal processing) . Thus, typical data never exactly fit the model that is beeing used, even when that model is correct. As statistical measure of goodness-of-fit the software provides the so called "reduced (weighted) chi-square" χ_r^2. Theoretically this value is around 1 for ideal fits, in practice values may be larger due to disturbances of the instrumental response and other systematic errors.

[1] O`Connor,D.V., Phillips, D.(1984), Time correlated single photon counting. Academic Press, London

2. Tutorial

The software described in this manual is designed to provide an easy access to the liftime information in each pixel and presents it in a user definable way as a color coded image. SPCImage 2.3 will run under all 32-bit Windows™ Operating Systems. A quick machine with at least 128 Mb of memory and a similar amount of free hard disk space is recommended. Screen resolution should be 1024x768 or better. The installation of the software is integrated in the *TCSPC package* and requires a licence key.

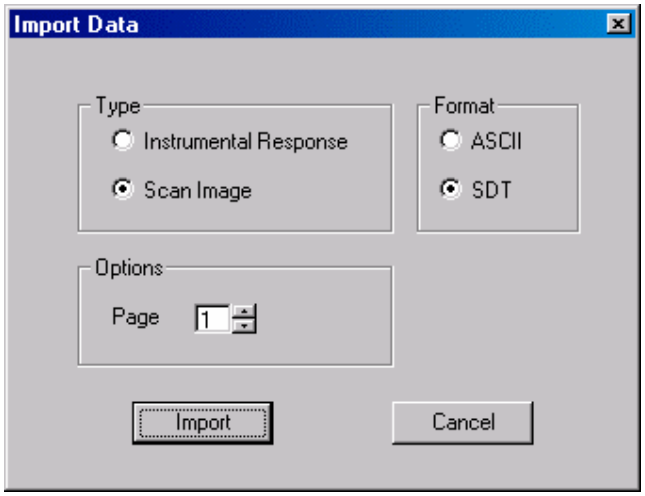
Figure 1 shows the color coding in combination with a conventional intensity image. It was taken from a single cell layer of fixed cells which were stained with two different dyes. Nonlinear least square fitting with a single-exponential decay delivered the fluorescence decay rates in the individual pixels of the image. The color coded image displays the fluorescence intensity as brightness and the fluorescence time as color. Since the algorithm comprises deconvolution from an instrumental response as well as automatic subtraction of the dark noise both time resolution and signal to noise ratio can be enhanced significantly. The quality of the fit is shown for one selected pixel. The fluorescence decay times are clearly distinguished and are displayed by red and green colour respectively.

With the new version a special data file format for lifetime images with the extension **".img**" is introduced. Files with this extension can be saved and loaded by the application. As a new feature the loading may be now invoked by double clicking of the file or by dragging it into the main window of the SPCImage software!

2.1 File → Import

Since SPCImage gets all the fluorescence lifetime data from the measurement software it is necessary to transfer the data between the two applications. The dataset from a SPC-700/730/830 module is loaded into the application by importing an .sdt file which was created by the measurement software.

Version 2.3 of SPCImage allows to select different *measurement pages* eventually contained in the measurement file. Pages are used to store subsequent measurements (see explanation in the SPC user manual) or for measurements in which the routing capability is used. Version 2.3 can process ".sdt" files with a format from **single file** up to 1024 x 1024 pixel.



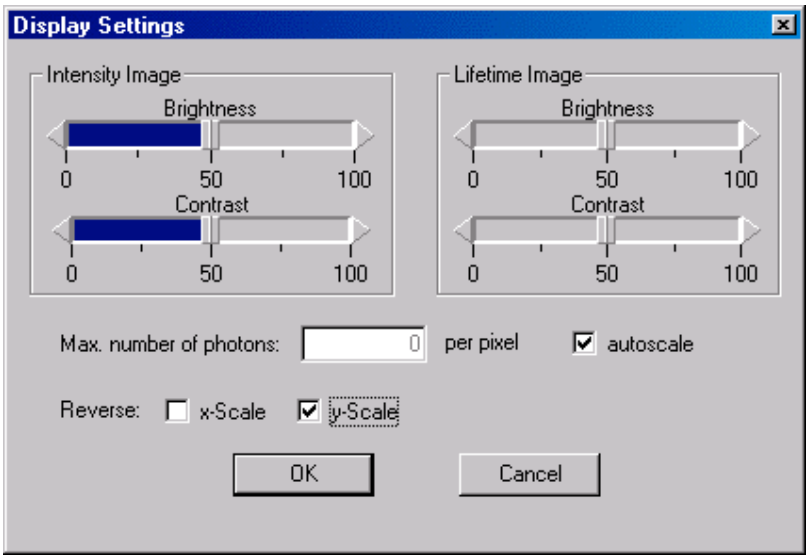
By default the file type is set to “Scan Image” to import the dataset of the image as described above. By switching to “Instrumental Response” the program will try to import a single curve containing the temporal behaviour of the measurement system (optics + electronics). A more detailed description of this procedure can be found below.

2.2 Options → Display Settings

An *intensity image* of the data is displayed after having successfully imported the data . This image is calculated from the time integrated number of counts for each pixel (*pixel-sum*). An autoscaling of the intensity is performed by selecting the range from 0 (black) to the maximum pixel sum within the image (white). There are two possible ways to change the intensity of the image:

i) When the “autoscale” checkbox is disabled a user defined maximum can be inserted. This feature enables an “absolute scaling” for comparing different measurements (or pages within one measurement).

ii) The brightness of the image can be controlled by the Brightness & Contrast Dialog. The sliders can be used to change the image appearance by “trial-and-error”.



2.3 Calculate → Instrumental Response (F5)

After importing the dat a decay curve and a fit will appear in the “single curve”-diagram at the bottom of the window. It belongs to the pixel which was selected with the small blue cursor within the intensity image. The model function of the fit curve is a “Multiexponential Decay” with offset-correction. The latter taking ambient light and/or dark noise into account:

$$F(t) = a_0 + a_1 e^{-t/\tau_1} + a_2 e^{-t/\tau_2} + a_3 e^{-t/\tau_3}$$

The number of exponential decays that are used is given by the “Components” parameter in the lower right of the application window:

The constant a_0 is denoted as "Offset" and can be fixed, i.e. held at a user given value, by activating the check box next to the value field. If the offset it is not fixed the program will calculate this parameter by averaging the number of photons in front of the first vertical cursor! Please note that the intensity coefficients a_1 through a_3 are given as *relative* amplitudes which can not be changed by the user. The decay-times τ_1 through τ_3 are denoted in picoseconds (ps) and are "fixable". If the "Fix"-box is checked the corresponding value must be inserted by the user and is not changed by the program during the fitting process.

The analytical function is convoluted with an "instrumental response" function before it is fitted to the data. By default the fitting algorithm uses a dataset for the convolution which is calculated from the first derivative of the rising part of the fluorescence. However, the *true* shape of the instrumental response is determined by the detector and is also influenced by the excitation source and the optical pathway of your system. Therefore the shape of the system response calculated by this procedure is only a rough approximation and may cause deviations especially in the first part of the curve. Please note that only the region *between* the two vertical lines is taken into account by the fitting procedure. Therefore it may appear that the fit outside these lines is not correct and the cursors have to be moved to the region of interest. It is a good idea to place the first cursor line directly in front of the rising edge of the fluorescence decay and the second near the end of the curve where a dropping of the decay not yet appears.

The *time-shift* parameter denoted as "Shift" determines how the instrumental response function is located relative to the rising edge of the fluorescence (given in channels). The scattering factor takes into account how much of the exciation light is directly scattered instead of being absorbed and emitted as fluorescence.

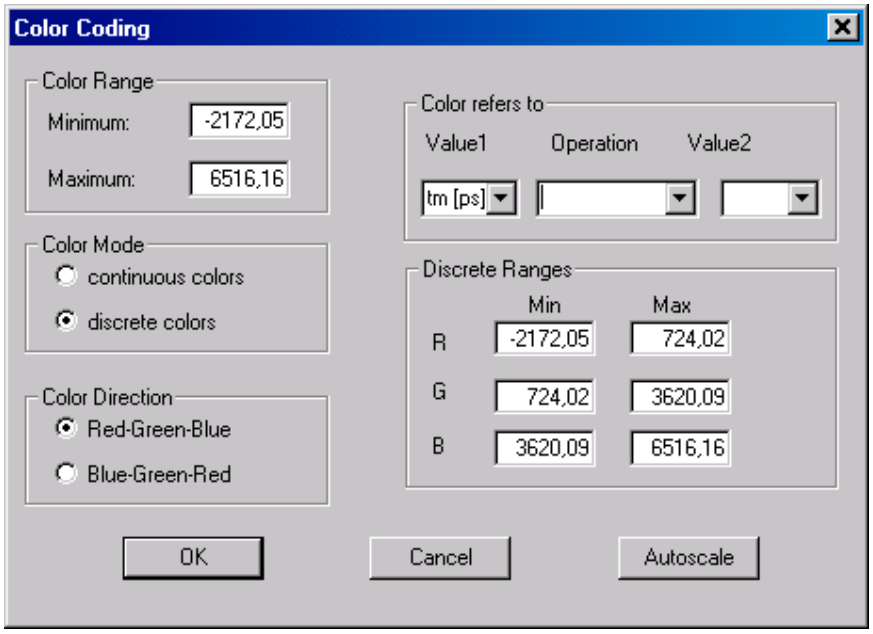
If the curve seems to have very few counts it is a good idea to increase the so called binning factor. This factor defines how many pixels are combined before the decay time is calculated. The preselected value of 1 means that (2 n + 1) x (2 n +1) = 9 pixels are summed up to produce one decay curve. Values greater than 3 are not recommended since a higher factor will decrease the spatial resolution. In addition you can select a " threshold". This value defines the lower limit of photons that at least has to be in the maximum of the curve - otherwise fitting is skipped.

2.4 Calculate → Decay Matrix (F2)

Now you can create a lifetime image by using the function to calculate the "Decay Matrix" which contains the calculated fit parameters for every pixel. This procedure needs most of the computational resources and takes several seconds to some minutes depending on model complexity, image size and computer speed.

2.5 Options → Color Coding

After calculating the decay matrix for the first time the color images might not revealing the full information. Therefore it is recommended to adjust the color range according to the particular lifetime distribution of the individual image. For this purpose please use first the "Autoscale"-button and then the vertical black cursors which are positioned at left and right side of the lifetime distribution panel respectively.



If the mode *Continuous Colors* is used the position of the black cursors are reflected by the *Color Range* values (Minimum/Maximum) in the upper left of the dialog box. In the upper right of the dialog three list boxes can be found which belongs to the *Displayed Value*. As a default the average lifetime "tm" of the decay matrix is taken. This means that for a multi-exponential decay the average lifetime is calculated according to the formular:

$$\tau_m = \frac{\sum_{i=1}^{N} a_i \tau_i}{\sum_{i=1}^{N} a_i}$$

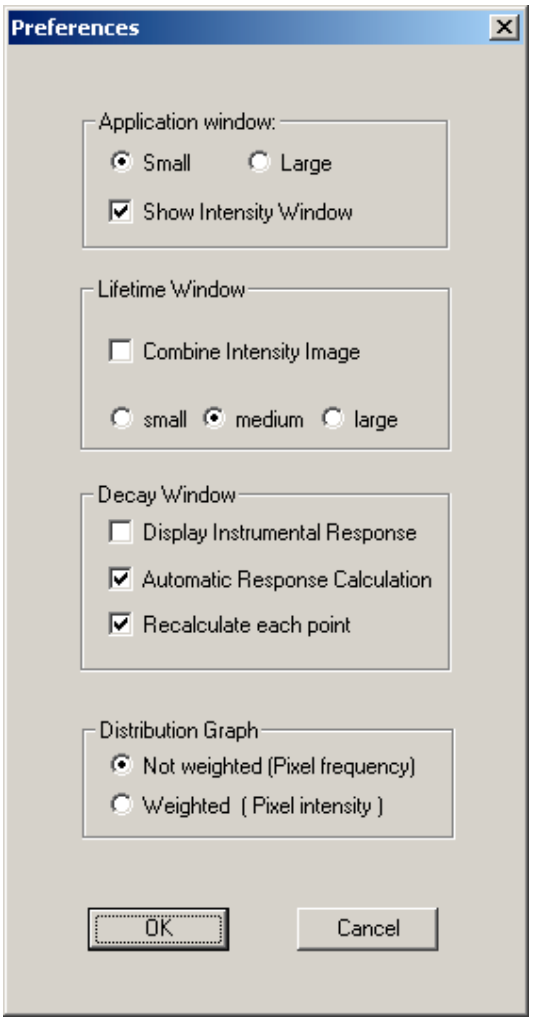
As the default mode for the color is a continuous color scale. The value τ_m is presented with a rainbow-like distribution which runs from red (small values) to blue (large values). By changing the *Color Direction* to "Blue-Green-Red" this order can be reversed. For another presentation the *Color Mode* may be switched to *Discrete Colors*. With this option it is possible to select the regions for red (R), green (G) or blue (B) manually.

Next to "tm" the *Value1* of the *Displayed Value* box can be choosen as the lifetime components: (t1, t2 , t3) the relative amplitudes (a1, a2, a3) or the relative qunatum yields (q1, q2, a3) of the individual components. In this case the color image will present only the selected value. of taking the lifetime average. Moreover the colour coded value can be the result of a simple arithmetic

calculation (sum, difference, product, quotient) selected by the operation selection box. Please note that the distribution curve in the color scale window (see below) will also change in this case since it always reflects the distribution of the value which is finally used to generate the color image.

2.6 Options → Preferences

It is possible to configure the general appearance of the application by this dialog. If the Windows™ operating system uses “large fonts” it is preferable to switch the application window also to “Large”.



By default the “Show Intensity Window”- checkbox is switch on. This means that the intensity image is presented next to the lifetime image. To safe space within the main window you may switch off this option. In this case the distribution graph is presented in a larger diagram to display more details.

Please note that the application window has to be restarted when changing some of the application window options! This is done automatically after pressing ok – do not restart the application manually during this process.

For all parts of the lifetime image where no fit parameters could be determind (due to low photon numbers or excluding by the range-of-interest cursors) it is possible to diplay the intensity information instead. When checking the “Combine Intensity Image” this option is enabled and you will usually see a part of the image to be gray-scaled. The group of radio button “small-medium-large” can be used to switch the resolution of the image windows. The “large” option can be helpful to display the details of high resolution images (512 x 512 and higher) wheras the “small” option might fit better for image resolutions (128 x 128 or lower).

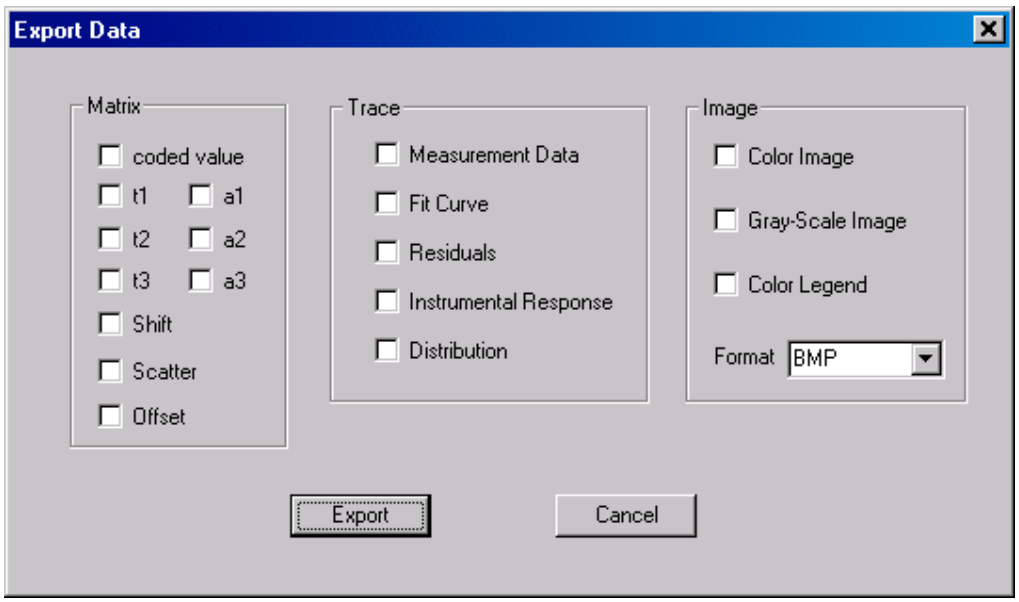
The “Decay Window” group allows to activate the response function inside the decay diagram. Furthermor it is now possible the switch of the automatic recalculation of the parameters within each pixel. This was implemented due to the following reason: If the “Recalculate each point” box is switched on (default) the software will repeat the fit for each pixel when moving the cursor or changing the parameters of the fit model. Whereas this guarantees that a correct fit is presented for each pixel it might not relect the decay matrix that was calculated before by pressing Calculate->Decay Matrix. If the the “Recalculate each point” checkbox is switched off the decay window always presents the of the original parameters of the decay matrix – only in the case the pixel was not fitted before it will present a new fit.

The “Automatic Response Calculation” which is switched on by default and enables a quick and robust estimation of the system function. It also places the first cursor directly in front of the rising part of the decay curve after the “Calculate -> Response Function (F5)” procedure is invoked. If the “Automatic Response Calculation” is switched off the first cursor has to be placed manually in front of the rising part of the fluorescence trace and the second near the maximum of the trace. In this case the the binning factor should be increased to get a trace with a well defined rising edge. Please note that the “Calculate -> Response Function (F5)” command takes only the region between the cursor lines into account when working without “Automatic Response Calculation”.

The Display of the

2.7 File → Export

The new version comes with a convenient export filter to enable postprocessing of the generated data by an external software. The dialog box shown below lists all data elements that may be



exported by clicking the checkboxes next to it.

All data generated by the Calculate->Decay Matrix command can be stored into ASCII files with the help of the first group *Matrix*. After pressing *Export* the program asks for a name of the export file. Please note that the name contained in the dialog box will be used as "ending" of the complete filename. The first part is automatically added by the program according to the type of data.

The created files for the *Matrix* contain space seperated values with an end-of-line character for each row. The "coded value" matrix contains the values from which the current color image was generated (2.5 Options → Color Coding). The *Trace* group allows to export the data of single curves. The format of these files is x-Value and y-Value in each row seperated with a space.

Two image formats are supported by the new version. Next to the windows bitmap format (.bmp) it is now possible to choose the Tagged Image File Format (.tiff) for the color- and the gray-scale image. In addition to this it is possible to export the color legend bar as an extra file. This will allow to present the correct color coding together with a lifetime image.

Complete reconstruction of tissue structures and optical properties from time-resolved data is extremely demanding and not entirely solved yet. Nevertheless, it turns out that a large number of time-resolved detection channels is required to obtain meaningful optical tomographic images. For in-vivo applications it is important to keep the measuring time short, i.e. simultaneous recording at different projection angles is highly desired. An additional advantage of a multi-channel measurement is that for a given number of photons injected the total number of detected photons is increased improving signal-to-noise ratio compared to single-channel detection.

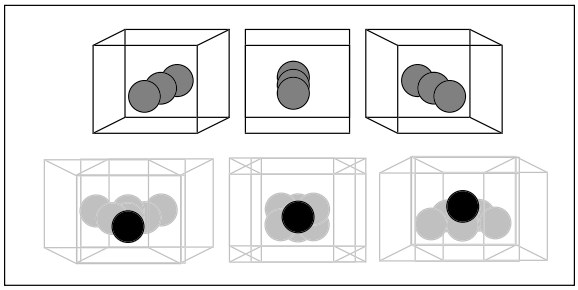


Fig. 1: Appearance of an object under different projection angles (top) and tomosynthesis (bottom)

To record data for optical mammography containing temporal information, modulation techniques and time-correlated single photon counting techniques are used. TCSPC has the benefit of a higher system bandwidth limited by the transit time spread of the detector rather than by the width of its pulse response. Furthermore, TCSPC yields a shot-noise limited signal-to-noise ratio and a near-ideal sensitivity. Conventional TCSPC electronics is, however, too bulky for the required number of channels and poses severe restrictions on the photon count rate.
We present improved TCSPC electronics which can be used for up to 32 detector channels with an effective overall count rate of 20 MHz and demonstrate its application to optical mammography.

2. HIGH COUNT RATE MULTICHANNEL TCSPC

The TCSPC device is shown in fig. 2, left. It is a package of four completely parallel TCSPC channels. Each channel can be expanded to record the signals of eight detectors simultaneously and has a maximum useful count rate of 5 MHz. The package is operated in a standard PC. The block diagram of one TCSPC channel is shown in fig. 2, right.

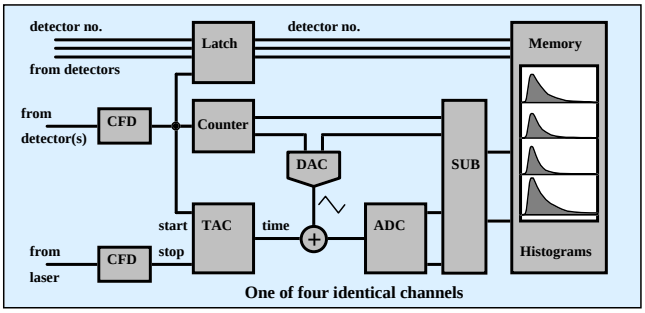


Fig. 2, left: The complete 4-channel device ready to be inserted in a PC
right: Block diagram of one TCSPC channel. CFD - constant fraction discriminator, TAC - time-to-amplitude converter, DAC - digital-to-analog converter, ADC - analog-to-digital converter, SUB - subtraction circuit

Basically, the device uses the well-known reversed start-stop principle with constant fraction discriminators, CFD, time-to-amplitude converter, TAC, and analog-to-digital-converter, ADC, that addresses a memory in which the histogram of the photon density over time is built up.

High count rate multichannel TCSPC for optical tomography

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ABSTRACT

An improved Time-Correlated Single Photon Counting (TCSPC) technique features high count rate, low differential nonlinearity and multi-detector capability. The system has four completely parallel TCSPC channels and achieves an effective overall count rate of 20 MHz. By an active routing technique, up to eight detectors can be connected to each of the TCSPC channels. We used the system to record optical mammograms after pulsed laser illumination at different wavelengths and projection angles.

Keywords: (170.3660) light propagation in tissue; (170.6960) tomography; (170.3830) mammography

1. INTRODUCTION

Attempts to image structures in deep tissues by optical tomography based on near-infrared (NIR) light are faced with the problem that details are washed out by the strong scattering of photons. Therefore, optical tomography of thick tissues cannot achieve spatial resolution comparable to that of X ray methods. There are, however, two benefits of optical methods: At sufficiently low power NIR radiation does not harm tissue. From absorption coefficients measured at several wavelengths, physiological quantities can be derived, in particular haemoglobin concentration and blood oxygen saturation [1].

Light propagation in tissue is governed by scattering and absorption of photons. Unfortunately, these effects cannot be reliably distinguished in simple steady state measurements. However, when pulsed light is used, time-resolved detection of diffusely transmitted or reflected intensity yields additional information. Although increased scattering and increased absorption both decrease the output intensity, stronger scattering broadens the transmitted or reflected pulse while increased absorption tends to narrow it. By modelling propagation of light as diffusion of photons and by using appropriate boundary conditions when solving the diffusion equation, the reduced scattering coefficient and the absorption coefficient of a homogeneous medium can be distinguished and quantified using the shape of the broadened pulse only [2].

Optical tomography aims at the detection of inhomogeneities in tissue and relies on measurements at a number of detector positions for each source position. Various mathematical methods have been developed to reconstruct the position and the optical properties of inhomogeneities from measured data [3, 4]. Time-resolved techniques improve localisation and characterisation of inhomogeneities [5], as information on the pathlength of each photon becomes available. Inhomogeneities differing from the surrounding tissue by scattering and absorption have different influence on the distribution of times of flight of photons.

Transillumination scanning optical mammography [6] is performed in slab geometry, i.e. the breast is compressed between two glass plates with source and detector, positioned on opposite sides, scanned synchronously. Detection at different projection angles gives additional information on the internal structure of the tissue. The apparent location of the image of structural details depends on the depth of the corresponding structure in the tissue. By shifting and adding images obtained at different projection angles structural details in selected layers can be enhanced while those of other layers are smeared out (fig.1). The method is known as “digital tomosynthesis” in X ray mammography [7] and requires data taken at a sufficient number of projection angles. This empirical approach yields images containing depth information but cannot compensate for the loss in resolution and contrast due to scattering.

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In contrast to the conventional setup, we use an ultra-fast ADC with an error reduction circuitry and an extended memory structure for multidetector operation [8]. The applied principle of analog to digital conversion is shown in fig. 2. The ADC is supplemented by an up-down counter which counts the incoming photon pulses. The counter output data is fed to a DAC generating a triangle signal which is added to the output of the TAC. The ADC converts the sum of both signals, giving the sum of the TAC value and the counter data. At the output of the ADC, the counter data is subtracted from the ADC result. This restores the original detection time measured by the TAC. Compared to a direct ADC conversion, this principle has a striking benefit: It converts the time of each photon in a different place on the ADC characteristics and therefore smoothens out the ADC errors. This smoothing works so efficiently, that we can use an ultra-fast ADC with only 12 bit non-missing code accuracy. Together with a speed-optimised TAC, we achieve a signal processing time of only 125 ns per photon.
The effect of the ADC error reduction is shown in fig. 3, left. An unmodulated light signal was recorded without error correction, and for a 7 bit and 9 bit width of the counter. Fig. 3, right, shows that the instrument response function is not substantially broadened by the error reduction.

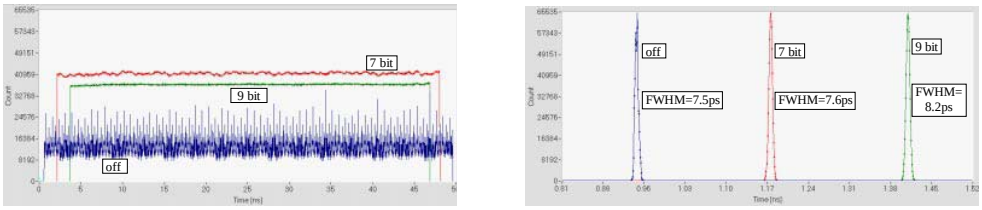

Fig. 3, left: Unmodulated light recorded without error reduction, and with a counter data width of 7 bit and 9 bit
right: Corresponding instrument response function for an electrical test signal

Fig. 4 shows how one TCSPC channel is expanded for operation with eight detectors. The expansion is based on the fact that it is unlikely to detect several photons in the same laser pulse period. This is a general condition for any TCSPC measurement and therefore not a restriction for the multi-detector configuration.
The single photon pulse from each detector is fed to a discriminator that responds when the detector has seen a photon. The discriminator outputs are encoded to generate a 3 bit channel number.
The output signals of all detectors are merged into one output line by a summing amplifier. Thus, on the detection of each photon we get a detector pulse and a detector number.
The detector pulse is connected in the usual way to the CFD input of one TCSPC channel (fig. 2). When the TCSPC channel detects this pulse, it writes the 'channel number' bits into a data latch that controls the memory segment in which the photon is stored. Thus, in the TCSPC memory eight histograms corresponding to the individual detectors are built up.
In the unlikely case that several detectors respond in the same laser pulse period, the encoder delivers an 'invalid' signal which inhibits the storing of the current photon. Therefore, the well-known pile-up distortion of the histogram is even smaller than with a single detector operated at the overall count rate of the eight detectors.

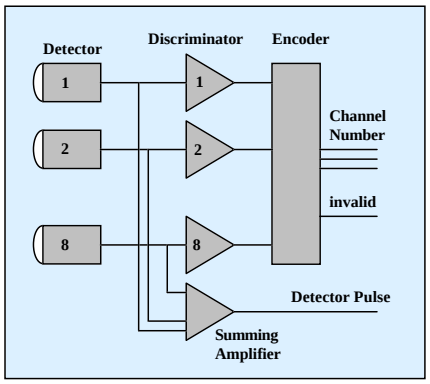

Fig. 4: Expansion for multi-detector operation

3. APPLICATION TO LASER-PULSE MAMMOGRAPHY

The TCSPC device was tested in the laser-pulse mammograph described in [6]. For the experiments described below, the mammograph was upgraded to four detection channels. When recording mammograms, the source-detector arrangement is scanned across the slightly compressed breast in two dimensions. Time-resolved transmittance is measured within 100 ms at each of 1000-2000 scan positions, 2.5 mm apart. Mammograms are recorded within 3-6 min. The diameter of the illuminated spot at the upper surface is about 3 mm, the detector fibre bundles are 4 mm in diameter.
All detectors were Hamamatsu R7400U-02 photomultipliers. Preamplifiers (50db) were used to compensate for the relatively low gain of the R7400U-02 detectors. The arrangement of the detector fibre bundles D1 to D4 is shown in

fig 5 (upper right). D1 was the direct channel opposite to the source fibre, D2, D3 and D4 were offset by 2 cm. Picosecond laser pulses from a 670 nm and a 785 nm diode laser (PicoQuant, Berlin) were multiplexed.
To test the system we used the phantom shown in fig. 5 (upper left). It consisted of a rectangular cuvette with several black wires of 1.7 mm diameter and one transparent and three black spheres of 8 mm diameter. The wires were arranged at different depths and orientations. The two longest wires ran parallel to and 0.6 cm apart from the front and rear surfaces of the scattering liquid, respectively. The black spheres were positioned 1.2 cm from the front plane, at the centre plane and 1.2 cm from the rear plane. The transparent (glass) sphere was placed at the centre plane. The inner thickness of the cuvette was 6.8 cm. As scattering liquid a mixture of whole milk and water with addition of a small amount of black ink was used. At 670 nm the reduced scattering and absorption coefficients were about 10 cm^{-1} and 0.04 cm^{-1}, respectively, and thus typical of the optical properties of breast tissue.
Fig. 5 shows the result of a 65 by 53 pixel scan with a step size of 2.5 mm. The images were created from the time-of-flight distributions of the photons integrated over time and normalised at a common reference position. The different appearance of the phantom under different projection angles is clearly visible. The image coordinates are referenced to the coordinates of the source. The objects close to the source (lower sphere and wire running from upper left to lower middle of the image, s. fig. 5) do not change their position at different viewing angles. The other objects (e.g. upper sphere and wire running from lower left to upper middle) appear shifted depending on their depth and on the detector position.

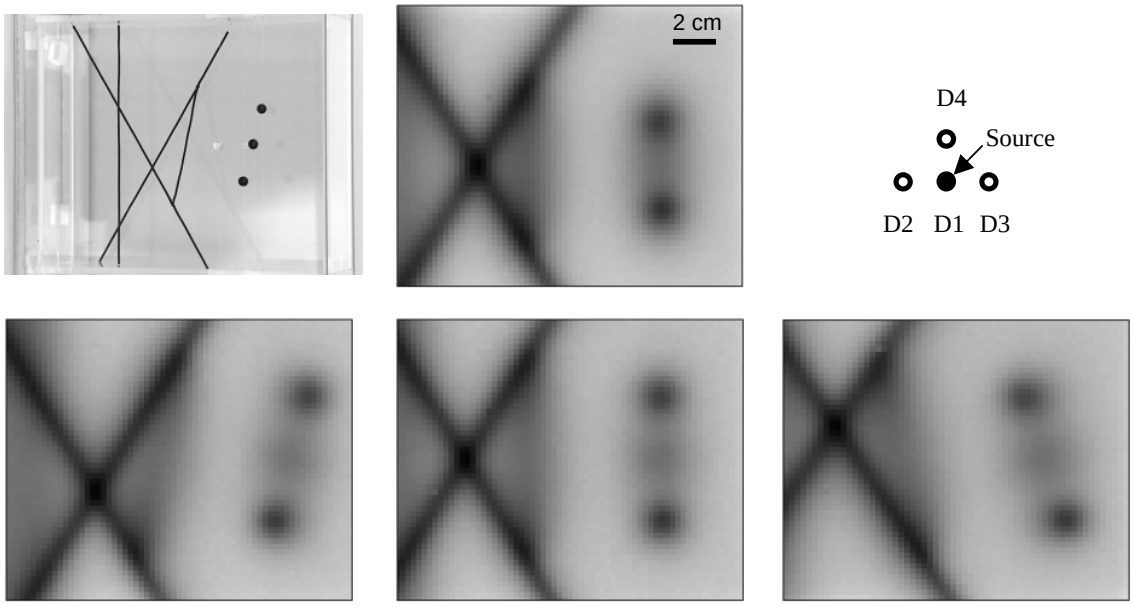

Fig. 5: Phantom images - arranged according to the positions of the detecting fibre bundles (s. upper right) - representing total photon counts for different projection angles. In all figures a linear gray scale is used. Black corresponds to the minimum, white to the maximum value of the photon counts displayed. A photo of the phantom taken from an oblique direction is shown (upper left).

Figs. 6b and c show the results of tomosynthesis [7, 6] based on the images corresponding to detector positions D1 to D3. For comparison, the image corresponding to D1 (s. fig. 5) is also included (fig. 6a). The raw images were added with appropriate offsets to synthesise images corresponding to a plane 0.8 cm below the source (fig. 6b) and a plane at the centre of the phantom (fig. 6c). The images show the black objects (wires and spheres) of the phantom more or less clearly. The contrast of objects in the synthesised plane remains unchanged while the contrast of objects in other planes is smeared out. There is, however, no indication of the glass sphere at the centre plane, not even in the corresponding tomosynthesis image.

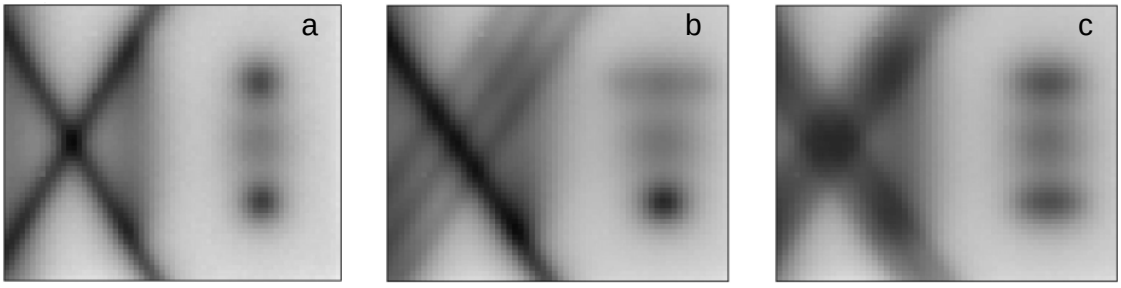

Fig. 6: Tomosynthesis for localisation of objects at various depth using images representing total photon counts (s. fig. 5): Raw image derived from the central detector D1 (a), tomosynthesis of images recorded by detectors D1, D2 and D3 for a plane close to the source (b) and for the central plane (c).

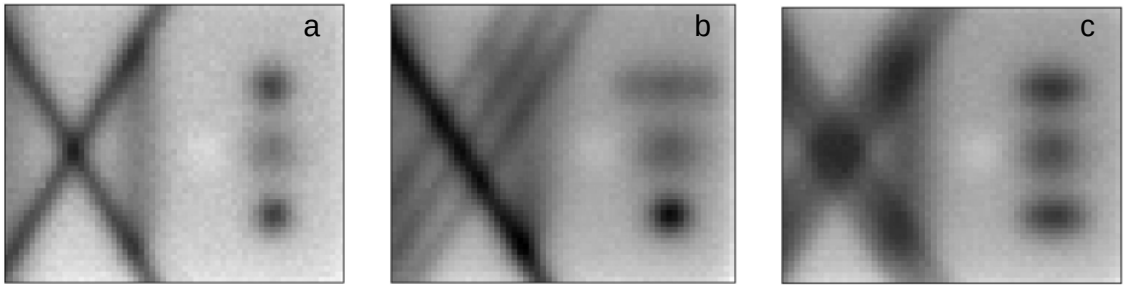

Fig. 7: Tomosynthesis of images generated from photons in an early time window: Raw image derived from the central detector D1 (a), tomosynthesis of images recorded by detectors D1, D2 and D3 for the same planes as in fig. 6 (b, c).

Fig. 7 was obtained from photon counts in an early time window of the times-of-flight distribution of photons. The window was adjusted to contain 10% of all photons detected at a reference position. Although the images contain some noise due to the smaller number of photons, the glass sphere shows up. Adding images appropriately shifted for tomosynthesis enhances not only the objects in the synthesised plane but also reduces the noise. It should be noted that three projection angles are sufficient to triangulate the depth of an inhomogeneity of any shape. However, to obtain images of selected planes a much larger number of projection angles must be used.

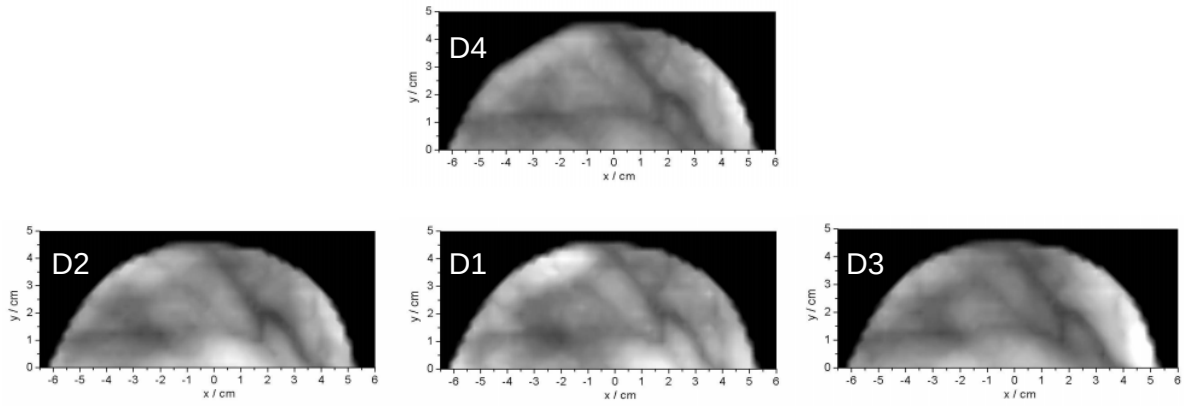

Fig. 8: Mammograms of a volunteer recorded simultaneously at four projection angles. The images were generated from photon counts in a late time window. The arrangement of the mammograms corresponds to that of the detectors D1-D4 (s. fig. 5).

Fig. 8 shows 51 by 21 pixel mammograms of a breast of a volunteer recorded at 785 nm by the four-channel setup. The mammograms were obtained by using photon counts in the 8th of 10 consecutive time windows each containing 10% of all photons detected at a reference position. It was shown previously that mammograms based on this time window are essentially free of edge effects without requiring correction algorithms to be applied [6]. The mammograms of fig. 8 corresponding to the different projection angles show slight differences. Since the pattern of the superficial blood vessels does not change significantly with the projection angle it can be concluded that these vessels were close to the source plane.

4. CONCLUSIONS

The TCSPC device described above has successfully been used to record time-resolved transmittance of phantoms and a compressed breast at high speed, high accuracy and high time resolution.

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Picosecond Fluorescence Lifetime Microscopy by TCSPC Imaging

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Abstract

A new Time-Correlated Single Photon Counting (TCSPC) imaging technique delivers combined intensity-lifetime images in a two-photon laser scanning microscope. The sample is excited by laser pulses of 150 fs duration and 80 MHz repetition rate. The microscope scans the sample with a pixel dwell time in the µs range. The fluorescence is detected with a fast PMT at the non-descanned port of the laser scanning microscope. The single photon pulses from the PMT and the scan control signals from the scanning head are used to build up a three-dimensional histogram of the photon density over the time within the decay function and the image coordinates x and y. Analysis of the recorded data delivers images containing the intensity as brightness and the lifetime as colour, images within selected time windows or decay curves in selected pixels. The performance of the system is shown for typical applications such as FRET measurements, Ca imaging and discrimination of endogenous fluorophores or different dyes in living cells and tissues.

Keywords: Fluorescence lifetime imaging, laser scanning microscope, time-correlated single photon counting

1. Introduction

To investigate molecular interactions in cells and subcellular structures fluorescence markers are used which specifically link to protein structures. Staining the sample with different dyes and recording the fluorescence image reveals the cell structures via the different fluorescence spectra and fluorescence lifetime of the dyes. Energy transfer between the dye molecules and the proteins changes the fluorescence quantum efficiency and thus the fluorescence lifetime. Due to the variation of the dye concentration these effects cannot be distinguished in simple intensity images. Therefore, recording time-resolved patterns of the full fluorescence decay functions rather than simple intensity imaging is required to investigate molecular interactions in biological systems.

Recording time-resolved fluorescence images can be achieved by combining a confocal laser scanning microscope, a femtosecond Titanium Sapphire (TiSa) Laser and an advanced Time-Correlated Single Photon Counting (TCSPC) imaging technique.

2. The Laser Scanning Microscope

The optical principle of a confocal microscope is shown in fig. 1.

The laser is fed into the optical path via a dichroic mirror and focused into the sample by the microscope objective lens. In the traditional one-photon setup the light from the sample goes back through the objective lens, through the dichroic mirror and through a pinhole in the upper image plane of the objective lens. Light from outside the focal plane is not focused into the pinhole plane and therefore substantially suppressed. X-Y imaging is achieved by optically scanning the laser spot over the sample, Z imaging (optical sectioning) is possible by moving the sample or the microscope up and down.

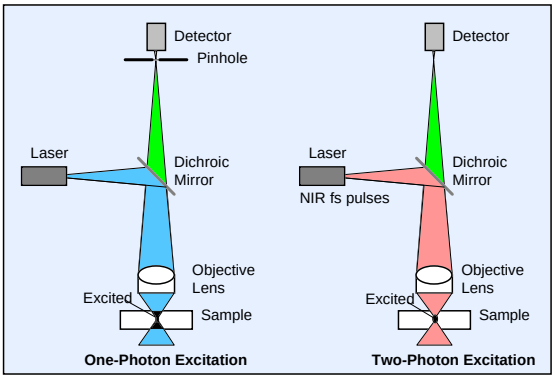

Fig. 1: Principle of a laser scanning microscope (scanning setup not shown)

With a fs TiSa laser the sample can be excited by two-photon absorption. Due to the short pulse duration and the small diameter of the Airy disk the photon density in the focus is very high, so

that the two-photon excitation works with high efficiency. Excitation occurs only in the focus, so that no pinhole is required to reject light from outside the focal plane. For imaging biological samples, two-photon excitation has several benefits:

- Scattering and absorption coefficients at the near infrared excitation wavelength are small. Therefore, layers deeply in the sample can be excited.
- If deep sample layers are imaged the scattering at the emission wavelength broadens the luminescent spot seen by the microscope objective. The absence of a pinhole allows to efficiently record the emitted light from a relatively large area while maintaining the optical sectioning capability.
- Living cells are surprisingly stable when illuminated with NIR radiation [1].

2. TCSPC Lifetime Imaging

2.1. Light Sources

Fluorescence Lifetime Imaging requires a pulsed excitation source. Two-photon microscopes use fs pulsed Ti:Sa lasers so that a suitable light source is already present. For one-photon excitation, a frequency-doubled Ti:Sa can be used. Synchrotron radiation has also been used [2], but is available only in some special cases. A reasonable cost solution are pulsed diode lasers which are available for the blue and red spectral range. These lasers deliver pulses down to 40 ps fwhm with up to 80 MHz repetition rate. They are by far a better solution than modulating the continuous laser of a commercial one-photon microscope which is sometimes suggested.

2.2. Detection Electronics

To record fluorescence images with lifetime information gated image intensifiers, modulated image intensifiers or single channel modulation techniques [3], gated photon counting [4], and time-correlated photon counting can be used.

In conjunction with a scanning microscope, most of these methods have serious drawbacks. Simple gating of the detector electronics discards the majority of the fluorescence photons and thus yields a poor sensitivity. Gated photon counting and single channel modulation techniques usually have problems with the high scanning speed of the microscope. Image intensifiers and other direct imaging techniques do not only record photons from the scanned spot of the sample, but also the thermal background events from the remaining detector area.

Gated photon counting can be accomplished in several parallel channels with subsequent time windows [4] . However, correct signal analysis is a problem if, as usual, the detector response shows afterpulses and bumps or if the optical system is not absolutely free of reflections.

Time-correlated single photon counting (TCSPC) is often believed to be an extremely slow method unable to reach short acquisition times. This ill reputation came from older NIM systems used in conjunction with low repetition rate light sources. Nevertheless, an early application of a high count rate TCSPC system to laser microscopy used an Ar+ laser for excitation and reached a count rate of $2*10^5$ / s [5]. State-of-the art TCSPC systems reach count rates in the MHz range and therefore are able to record decay functions within a few ms. The TCSPC method has a high detection efficiency, a time resolution limited only by the transit time spread of the detector and directly delivers the decay functions in the time domain. Furthermore, the TCSPC method can be combined with a multiplexed detection which is ideally suited for scanning applications [6].

The principle our TCSPC Lifetime Imaging module is shown in fig. 2.

The module employs an advanced TCSPC technique featuring both high count rate and low differential nonlinearity. It contains the usual building blocks (CFDs, TAC, ADC) in the

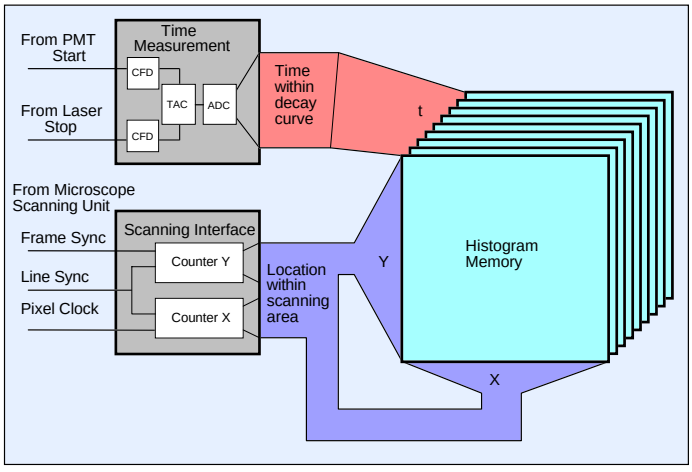

Fig. 2: Principle of TCSPC Lifetime Imaging

'reversed start-stop' configuration together with a scanning interface and a large histogram memory integrated on one board.

The TCSPC module receives the single photon pulses from the photomultiplier (PMT) of the microscope, the reference pulses from the laser and the Frame Sync, Line Sync and Pixel Clock signals from the scanning unit of the microscope. For each PMT pulse, i.e. for each photon, the TCSPC module determines the time of the photon within the laser pulse sequence and the location within the scanning area. These values are used to address the histogram memory in which the events are accumulated. Thus, in the memory the distribution of the photon density over X, Y, and the time within the fluorescence decay function builds up. The result can be interpreted as a two-dimensional (X, Y) array of fluorescence decay curves or as a sequence of fluorescence images for different times (t) after the excitation pulse.

Due to memory size limitations the maximum number of time channels per pixel and the minimum time channel width depend on the image resolution. Some possible combinations are given in table 1. For an image resolution smaller than the resolution of the microscope scan, several adjacent pixels are binned into one pixel of the TCSPC image.

image resolution pixels	t channels per pixel	min. t channel width ps
256 x 256	64	52
128 x 128	256	13
64 x 64	1024	3.25
32 x 32	4096	0.82
1024, line scan	4096	0.82

Table 1: Number of time channels and minimum time channel width for different image resolution

The actual time resolution depends on the detector and is 300 ps to 500 ps (fwhm) with the PMTs typically built-in in the microscope, and 30 ps (fwhm) with external Microchannel Plate (MCP) detectors. Interestingly, there is practically no loss of photons in the TCSPC imaging process. Due to the short dead time of the TCSPC imaging module (180 ns) nearly all detected photons are processed and accumulated in the histogram.

3. The TCSPC Scanning Microscope

The general setup of the TCSPC microscope is shown in figure 3. A Ti:Sa laser delivers femtosecond pulses in the wavelength range from 780 nm to 900 nm. We used different Coherent and Spectra Physics systems with a pulse width from 90 fs to 800 fs. The repetition rate was 76 or 82 MHz.

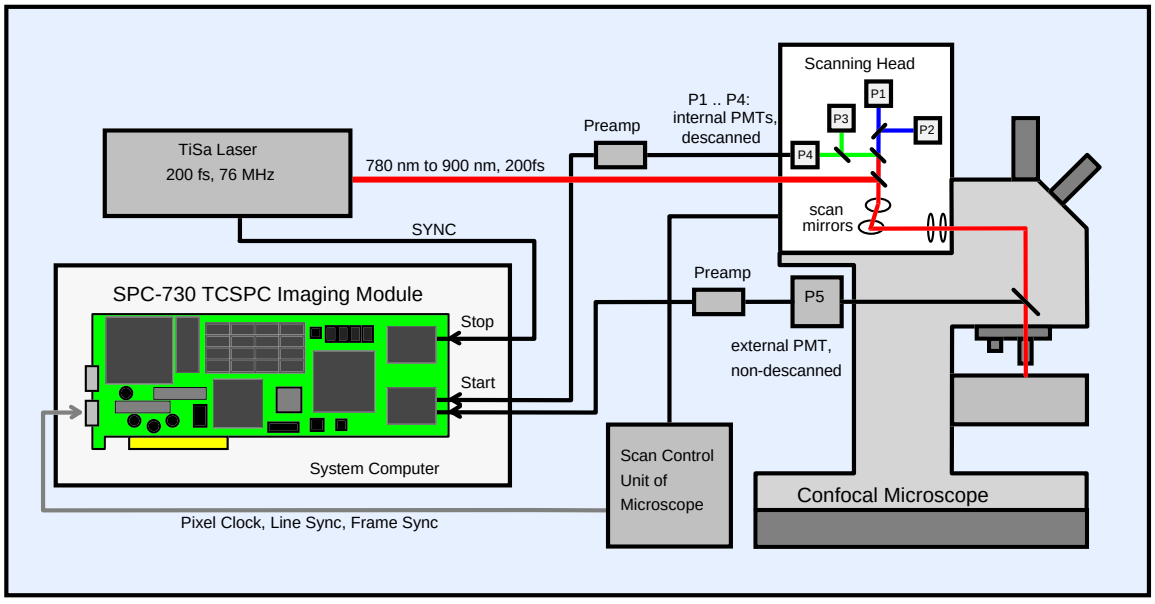

Fig. 3: General setup of the TCSPC Laser Scanning Microscope

The microscope scans the sample in the x-y plane providing an image of the sample in the focal plane of the objective lens. 3 D imaging is achieved by changing the depth of the focus in the sample. We used the Zeiss LSM-410, the LSM-510 in the Axiovert and the Axioplan version, and the Leica SP-1 and SP-2.

The scanning heads of these microscopes have several detection channels equipped with variable confocal pinholes, filters, and photomultipliers (PMTs). The PMTs are small side-window tubes which give good sensitivity but not the optimum time resolution in the TCSPC mode. Unfortunately, replacing these PMTs with faster ones is difficult if not impossible. To get a high time resolution from the detection channel of a LSM-510 scanning head we used a fibre in place of one detector and fed the light to a Hamamatsu R3809U-50 MCP PMT.

For two-photon excitation which does not require a pinhole, attaching a fast detector to the non-descanned port of the microscope is a better solution. We use the PMH-100-1 detector head of Becker & Hickl (transit time spread 150 ps) and the Hamamatsu R3809U-50 (transit time spread 30 ps). To get best performance from the R3809U-50 we use a Becker & Hickl HFAC-26-01 preamplifier. The instrument response functions for these detectors are shown in fig. 4 and fig. 5.

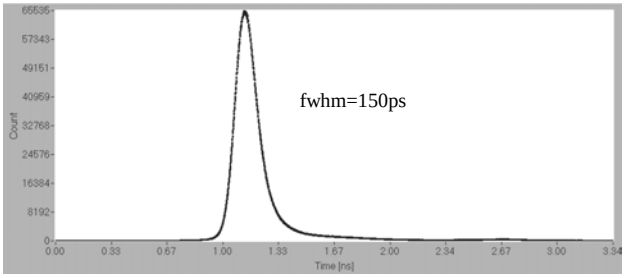

Fig 4: System response for the PMH-100 detector

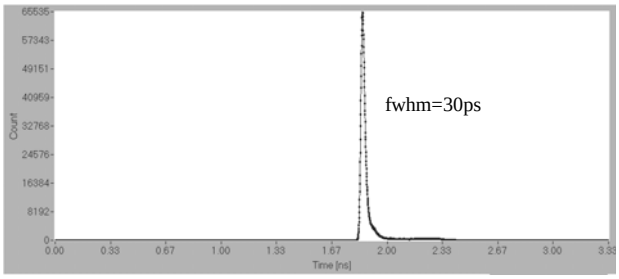

Fig 5: System response for the R3809U-50 MCP

If the non-descanned port is used problems can arise from incomplete blocking of scattered laser light. If the appropriate filters are not present in the microscope a suitable set of filters must be found and placed in front of the detector.

Data acquisition is accomplished by the Becker & Hickl SPC-730 TCSPC Imaging module [6]. The CFD input receives the single photon pulses of the PMT. Synchronisation with the laser pulse sequence is achieved by the SYNC signal from the reference diode of the Ti:Sa laser.

The SPC-730 module gets the scan control signals, Pixel Clock, Line Sync, and Frame Sync, from the scan controller of the microscope. Connecting the TCSPC module does not require any modifications in the microscope hardware and software. The scan parameters are controlled in the usual way via the microscope software. The normal scan speed, the zoom function, region of interest setting, etc. can be used also when recording lifetime images. Furthermore, the SPC-730 can be run in parallel with the standard image recording electronics of the microscope. Basically, steady state images and lifetime images in different spectral ranges can be obtained at the same time.

Due to the simple interfacing the SPC-730 TCSPC Imaging module can be adapted to almost any laser scanning microscope. The only requirement is that Frame Sync and Line Sync signals with TTL or CMOS levels can be made available. The Pixel Clock signal is not absolutely required. If it is not available a pixel clock can be generated in the SPC-730 module. The cost for upgrading a microscope is about $ 17,000 to $ 30,000 ($ 15,000 for the imaging module plus $ 2000 to $ 15,000 for the detector).

4. Results

Fig. 6 shows a TCSPC image of a single cell layer (double staining with Hoechst for DNA and Alexa 488 for Tubulin) obtained by simultaneous two-photon excitation at 800 nm in a Zeiss LSM-510 Axioplan microscope. The detector was a PMH-100-1 at the non-descanned port giving an instrument response function of 150 ps fwhm. The overall acquisition time was 60 seconds at a count rate of about 10^5 photons per second. The intensity image containing the photons of all time channels is shown left.

Deconvolution analysis delivers the fluorescence lifetime τ in the individual pixels of the image. This allows to generate intensity-τ images that display the fluorescence intensity and the fluorescence lifetime as brightness and colour (fig. 4, right). The quality of the fit is shown for two selected pixels (fig.4, bottom). The decay times of 2.0 ns and 2.8 ns are clearly distinguished.

Fig. 7 shows an intensity-τ image of the autofluorescence of human skin, obtained with two-photon excitation in a Zeiss LSM-410. The count rate was 25,000 / s. The two-photon fluorescence of melanin was found to be a potential tumor indicator [7]. Lifetime images can help to separate the melanin fluorescence from NADH or other fluorescence components.

Fig. 8 was obtained with a Leica SP-1 and shows a Ca lifetime image of cortex neurons after Calcium Green injection. The count rate was 10^5 / s. The lifetime of the Calcium Green is a direct measure of the calcium ion concentration. Therefore, lifetime images can avoid the intensity calibration normally used for Ca imaging.

Fig. 9 shows cells containing CFP and YFP excited at 820 nm. The count rate was 20,000 /s. The microscope was an LSM-410 Axiovert

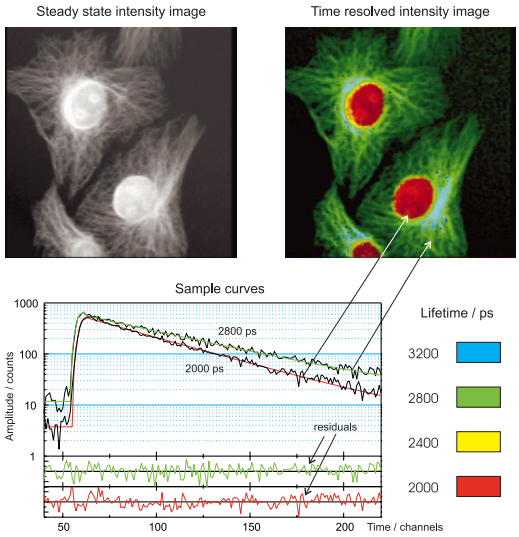

Fig. 6: Intensity image (top left), intensity-τ image (top right) and fitted curves

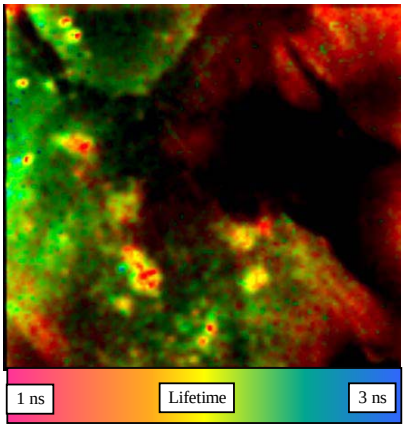

Fig. 7: Autofluorescence of human skin. Zeiss LSM-410, 2-photon excitation

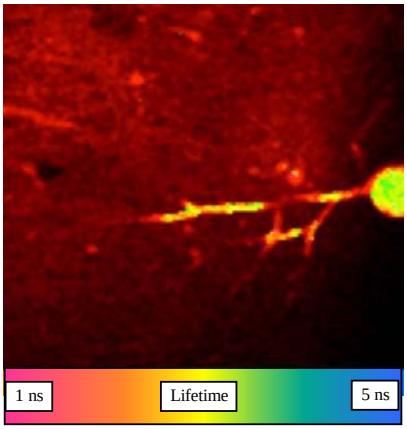

Fig. 8: Ca image of cortex neurons. Leica SP-1, 2-photon excitation

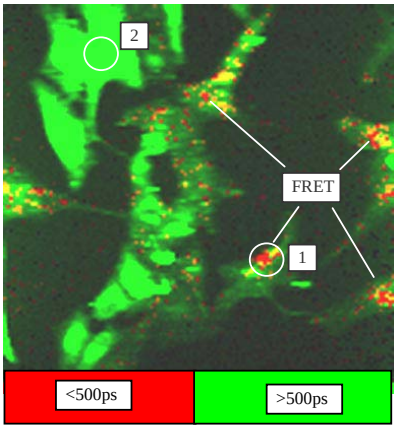

Fig 9: FRET in cells containing CFP and YFP. Zeiss LSM-410, 2-photon excitation

with a Hamamatsu R3809U-50 MCP attached to the non-descanned port. To suppress the excitation light and to select the fluorescence wavelength we used three 720 nm short pass filters (SWP-BL-720, Delta Light & Optics) and a 440 - 490 nm bandpass filter.

In this example, CFP acts as a donor and YFP as an acceptor for FRET. The CFP fluorescence is therefore quenched in the places where FRET occurs. The fluorescence in these places shows double exponential decay which cannot be displayed by a simple colour coding. Therefore, green was used for the short lifetime and red for the long lifetime component. The colours were mixed according to the relative intensities of the components. The decay curves of selected areas are shown in fig. 10.

The acquisition times for the lifetime images shown above were in the range from 30 seconds to 20 minutes. Although simple intensity images are obtained by TCSPC imaging in the same time as with the standard recording electronics of

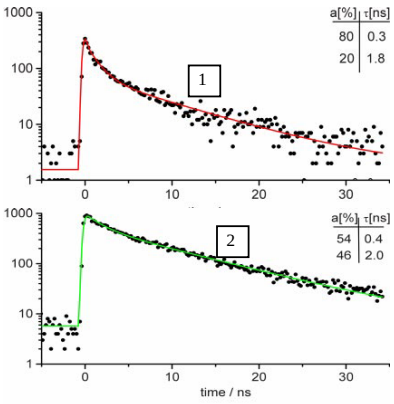

Fig 10: Decay functions of selected areas of fig. 9

the microscope, such images do not contain enough photons for lifetime analysis. Basically, the acquisition time could be decreased by increasing the laser power and thus the photon count rate. The SPC-730 module allows count rates up to some 10^6 photons per second which would decrease the acquisition time by a factor of 10 to 100. However, for living cells the limitation is the stability of the sample [1] which usually restricts the count rate to values less than 10^5 / s. Therefore, the only way to decrease the acquisition time is to confine the scanning area to a small region of interest. Since the acquisition time scales down with the number of pixels, reasonable data for an area in the order of 4 x 4 pixels can be obtained in less than 100 ms.

Conclusions

The results show the potential of TCSPC Laser Scanning Microscopy as a new method of fluorescence lifetime imaging. The field of application covers energy transfer measurements (Fluorescence Resonance Energy Transfer, FRET), Ca imaging, separation of multiple fluorescent labels, imaging of the autofluorescence of cells, and other fluorescence imaging applications of microscopic samples.

Acknowledgements

We are indebted to Thomas Behnisch, Istitut für Neurobiologie Magdeburg, Christoph Biskup, Klinikum der FSU Jena, Physiologisches Institut II, Zeiss Jena GmbH and Leica GmbH in Mannheim for cooperation and making available their microscopes and samples for the experiments.

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FRET Imaging by Picosecond TCSPC Laser Scanning Microscopy

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Abstract

A new Time-Correlated Single Photon Counting (TCSPC) imaging technique in conjunction with a two-photon laser scanning microscope was used to obtain FRET lifetime data from living cells. Double exponential decay analysis separates the FRET fluorescence from the fluorescence of the unquenched donor molecules. By using the intensity ratio of the quenched and the unquenched donor fluorescence images are created that show the size of the FRET in different regions of the cell.

Instrumentation

Recording of time-resolved fluorescence images was achieved by combining a confocal laser scanning microscope (Zeiss LSM-510 Axiovert), a femtosecond Coherent titanium sapphire laser and an SPC-730 time-correlated single photon counting (TCSPC) imaging module developed by Becker & Hickl, Berlin. The fibre output option of the LSM-510 was used to connect an ultra-fast MCP-PMT (Hamamatsu R3809U) for TCSPC detection.

The principle of the TCSPC imaging technique used in the SPC-730 is shown in fig. 1. The module contains the usual building blocks (CFDs, TAC, ADC) in the 'reversed start-stop' configuration together with a scanning interface and a large histogram memory integrated on one board. It receives the single photon pulses from the photomultiplier (PMT), the reference pulses from the laser and the Frame Sync, Line Sync and Pixel Clock signals from the scanning unit of the microscope. For each PMT pulse, i.e. for each photon, the TCSPC module determines the time of the photon within the laser pulse sequence and the location within the scanning area. These values are used to address the histogram memory in which the events are accumulated. Thus, in the memory the distribution of the photon density over X, Y, and the time within the fluorescence decay function builds up.

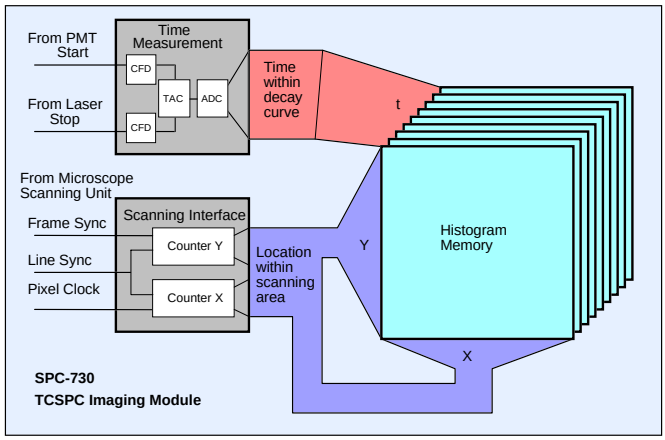

Fig. 1: Principle of the TCSPC Lifetime Imaging module

The data acquisition runs with the full scanning speed of the microscope. Due to the short dead time of the TCSPC imaging module (180 ns) there is practically no loss of photons in the TCSPC imaging process. The time resolution with the R3809U MCP is < 30ps (FWHM).

Results

Fig. 2 shows HEK cells containing CFP and YFP in the α and β1 subunits of the Na channels. Fig. 2a shows an intensity image of the donor fluorescence obtained by summing the photons from all time channels of the individual pixels. Fig. 2b shows an intensity-lifetime image built up from the fluorescence intensity as brightness and the average decay time obtained by single exponential analysis as colour. Fig. 2b clearly shows lifetime differences throughout the image of the cell.

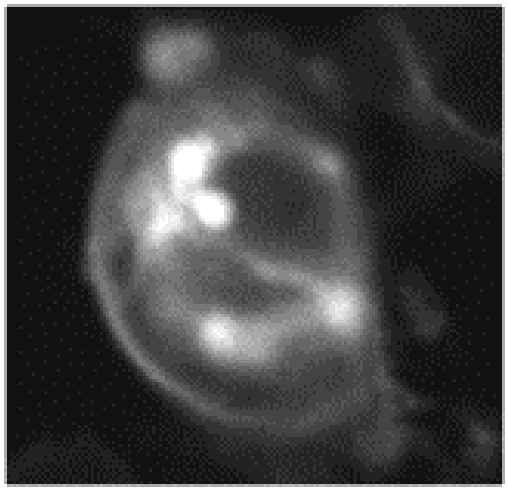

Fig. 2a: Intensity image of donor (CFP)

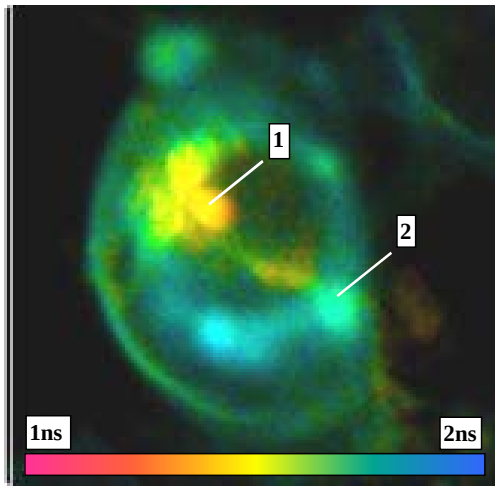

Fig. 2b: Intensity-Lifetime Image of Donor CFP

Fig. 3a shows the decay functions of selected pixels of fig. 2b. The decay is clearly not mono-exponential. Double exponential decay analysis reveals a fast lifetime component of about 0.37 ns and a slow component of 1.84 ns to 2.09 ns. The fast component most likely comes from the quenched CFP molecules while the slow component is fluorescence from the unquenched CFP and perhaps some YFP fluorescence. Both components are found anywhere in the image. However, the ratio of the intensity coefficients of the components differs considerably between regions with strong FRET and 'normal' regions (fig 3b).

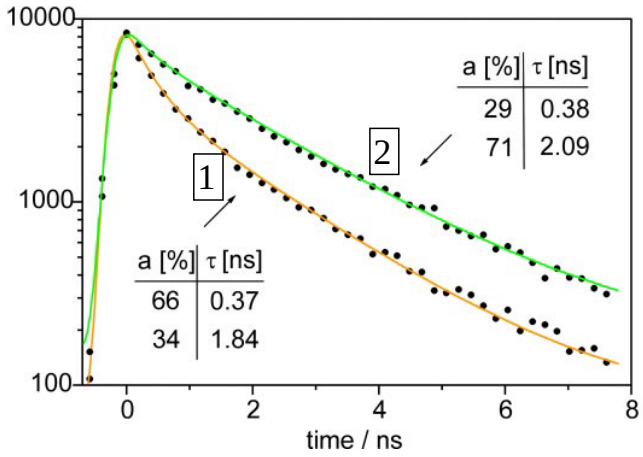

Fig. 3a: Fluorescence decay curves of selected pixels

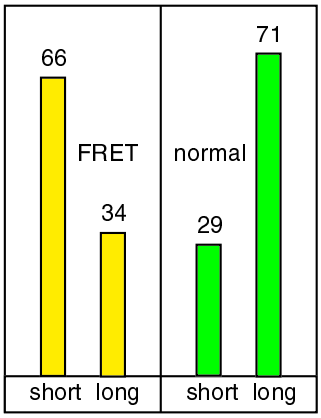

Fig. 3b: Relative intensity of fast and slow fluorescence component

Using the ratio of the intensity coefficients of the lifetime components as an indicator for FRET, we created imaged showing the size of the FRET as colour and the intensity as brightness. The result is shown in

fig. 4. It shows a lot of detail not visible in the pure intensity image and more contrast than the lifetime image obtained from the average lifetime.

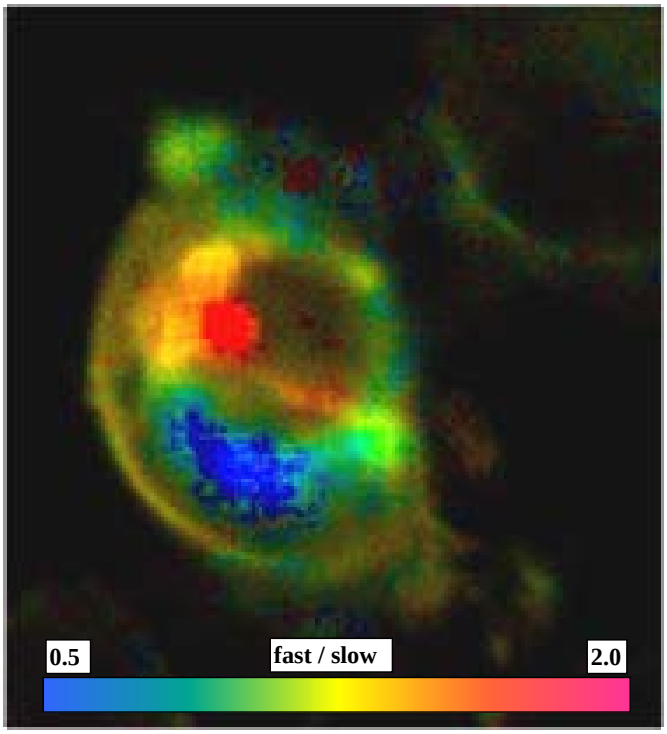

Fig. 4: FRET image built up from intensity (brightness) and ratio of coefficients of fast and slow decay component (colour)

Conclusions

A new TCSPC imaging technique in conjunction with a scanning microscope yields high quality fluorescence lifetime data. Applied to FRET in living cells, the technique delivers the decay components of the quenched and unquenched donor molecules. The ratio of the intensity coefficients of the quenched and the unquenched fluorescence component can be used to build up images that show the size of the FRET effect in the individual parts of the cell. The images show detail not visible in pure intensity images and better contrast than images created from the average lifetime.

Time-resolved detection and identification of single analyte molecules in microcapillaries by time-correlated single-photon counting (TCSPC)

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A PC plug-in card for on-line time resolved fluorescence detection of single dye molecules based on a new time-correlated single photon counting (TCSPC) module is described. The module contains all electronic components constant fraction discriminators (CFDs), time-to-amplitude converter (TAC), analog-to-digital converter (ADC), multichannel analyzer (MCA timers) on board required for TCSPC. A fast TAC design in combination with a fast flash ADC and an error-correcting ADC/MCA principle results in a maximum count rate of 8 MHz (dead time 125 ns). A dual memory architecture allows for unlimited recording of decay curves with collection times down to 150 μs without time gaps between subsequent recordings. Applying a short-pulse diode laser emitting at 640 nm with a repetition rate of 60 MHz in combination with a confocal microscope, we studied bursts of fluorescence photons from individual dye labeled mononucleotide molecules (Cy5-dCTP) in a cone shaped microcapillary with an inner diameter of 0.5 μm at the end of the tip. The flow of the conjugates was controlled by electrokinetic forces. The presented technique permits the counting and identification of all labeled analyte molecules present in a given sample due to their characteristic velocities, burst sizes, and fluorescence decay times. © *1999 American Institute of Physics.* [S0034-6748(99)03503-0]

I. INTRODUCTION

In recent years, several scientific groups achieved the capability to detect individual fluorescent molecules in liquids using laser induced fluorescence techniques.[1–10] Due to the improvements of the instrumentation, different dye molecules have been identified in solvents on the single-molecule level by their characteristic fluorescence decay times[11–15] and spectral properties.[4,16] Besides the implication of this highly sensitive detection techniques for the identification of various analyte molecules in diluted solutions, the fluorescence characteristics of individual dye molecules in specific local environments, i.e., the dynamic aspects, are of particular interest for different biological applications.[17–20]

In all applications of the single-molecule detection technique, it is desirable to count and identify each molecule present in a given sample with comparable efficiency. Therefore, great efforts have been made to construct a light barrier on the single-molecule level using laser induced fluorescence where all molecules have to pass.[21–23] Unfortunately, here a drawback is associated with the use of a femto liter detection volume which is generally applied in confocal fluorescence microscopy of single molecules. In order to direct the flow of all molecules through the detection area microcapillaries or -channels with inner diameters smaller than the detection area (<1 μm) have to be used. Unfortunately, a drawback is associated with the use of such small channels, namely strong adsorption of the analyte molecules to the wall due to the relatively great surface-to-volume ratio. On the other hand, if the adsorption can be efficiently suppressed by addition of detergents or additives, the use of such channels makes it possible to manipulate the motion of single molecules by electrokinetic, electro-osmotic, or capillary forces. Recently,[24] we were able to demonstrate the time-resolved identification of individual labeled mononucleotide molecules as they flow through a microcapillary with an inner diameter of 500 ± 200 nm. Furthermore, we showed that by addition of Tween 20, i.e., a nonionic detergent, adsorption of negatively charged analyte molecules to the capillary wall could be neglected. Hence, the flow of the negatively charged conjugates could be established by electrokinetic forces. Identification of the labeled mononucleotide molecules was performed by time-correlated single-photon counting (TCSPC) using a PC plug-in card with a minimum integration time of 600 μs and a dead time of several seconds between subsequent cycles of up to 128 histograms.

However, for on-line identification of each analyte molecule by the characteristic burst length, fluorescence decay time, and burst size a TCSPC-card with high acquisition rate and short integration times without dead times between sub-

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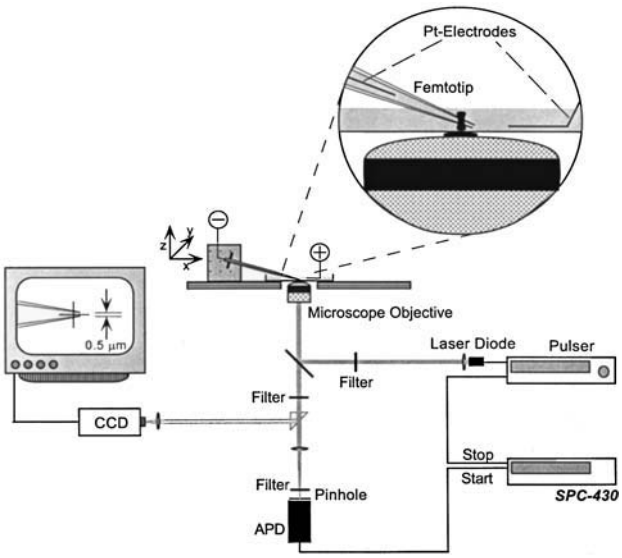

FIG. 1. Schematic diagram of the optical and electronic apparatus.

sequent cycles has to be used. In this article, we present time-resolved detection and identification of each labeled mononucleotide molecule Cy5-dCTP passing the detection volume by TCSPC in a microcapillary using a new developed PC plug-in card for TCSPC.

II. EXPERIMENT

A. Single-molecule apparatus and solvents

A schematic diagram of the used experimental setup is shown in Fig. 1. A pulsed diode laser (640 nm) served as excitation source. This system provided pulses of less than 400 ps full width at half maximum duration with a repetition rate of 60 MHz. The laser beam passes an excitation filter (639DF9; Omega Optics, Brattleboro, VT) and entered an inverse microscope (Axiovert 100 TV, Zeiss, Germany) through the back port and was coupled into an oil-immersion objective ($100\times$NA$=1.4$; Olympus, Tokyo, Japan) by a dichroic beam splitter. Measurements were done with an average laser power of 630 μW at the sample. The fluorescence signal was collected by the same objective, filtered by two bandpass filters (675DF50, Omega Optics, Brattleboro, VT; 680HQ65, AF Analysentechnik, Tübingen, Germany) and imaged onto a 100 μm pinhole oriented directly in front of the avalanche photodiode (AQ-131, EG&G Optoelectronics, Canada).

Measurements in open volumes were performed by using microscope slides with a small depression.[15] To ensure the detection of each analyte molecule, a cone shaped capillary with an inner diameter of about 0.5 ± 0.2 μm at the very end of the tip (Femtotip, Eppendorf-Nethler-Hinz GmbH, Hamburg, Germany) was used. The capillary dips into a tissue culture dish that contains pure glycerol. The small end of the capillary (ID$=0.5$ μm) was adjusted to meet the focus of the exciting laser beam by the use of a three-axis *(xyz)* electrostrictive actuator (20 nm resolution) and a charge coupled device camera. As solvent inside of the capillary, a 20 mM Tris-borate buffer *p*H 8.4 containing 65% water, 30% glycerol, and 5% Tween 20 was used. Tween 20 was added to prevent adsorption at the glass surface and to diminish the electro-osmotic flow.[25] Cy5-dCTP (Amersham Life Science, Braunschweig, Germany) served as analyte molecule and was used without further purification. Single-molecule solutions were prepared by diluting a 10^{-6} M stock solution with an appropriate amount of solvent down to the required concentration. To avoid blocking of the tip, all solutions were filtered through a 20 nm filter prior to injection into the femtotip. The flow of the negatively charged analyte molecules Cy5-dCTP through the femtotip was established by electrokinetic forces using two platinum electrodes. The cathode was inserted into the microcapillary and the anode was dipped into the solvent of the tissue culture dish.

B. TCSPC system

All time-resolved measurements were carried out in the reverse mode, i.e., the detector signal served as the start signal, whereas the laser pulse was used as the stop pulse. The instrument response function of the entire system was measured to be 420 ps.

1. Detector and synchronization channel

The principle of the TCSPC module SPC-430 is shown in Fig. 2. The single-photon pulses from the avalanche photodiode are fed to the input DETECT. To avoid an influence of amplitude jitter of the detector input pulses a constant fraction discriminator (CFD) is used to provide a well defined start pulse for the time-to-amplitude converted (TAC). The synchronization signals (stop pulse) generated by the pulse generator of the laser diode are fed to the input SYNC. To improve the timing of the synchronization signal also, the SYNC circuit contains a CFD. In addition, an adjustable frequency divider with ratios from 1:1 to 1:16 is implemented. Divider ratios greater than one can be used to record several periods of the light signal.

2. Time-to-amplitude converter

The TAC operates in the reversed start-stop mode. By this mode, the speed requirements of the TAC can be reduced because the cycle rate is controlled by the photon detection rate instead of the considerably higher repetition rate of the laser. The TAC output voltage is fed to a programmable gain amplifier (PGA). The PGA is used to amplify the interesting part of the TAC signal and can be adjusted by a gain and offset option so that the chosen part of the signal covers the complete ADC range. The speed limiting device is the PGA. Therefore, it is essential to have a very small settling time. The employed PGA has a settling time of less than 40 ns, even if the output voltage returns from saturation. To increase the effective count rate especially at high PGA gains, the output voltage of the PGA is checked by a window discriminator (WD) which rejects the processing of events outside of the selected time window. The TAC, the PGA, the WD, and the associated control circuits, as well as, the CFD and the SYNC circuit are integrated in compact hybrid circuits. The small size of all circuits reduces crosstalk between the start and the stop channel, which is essential to achieve a

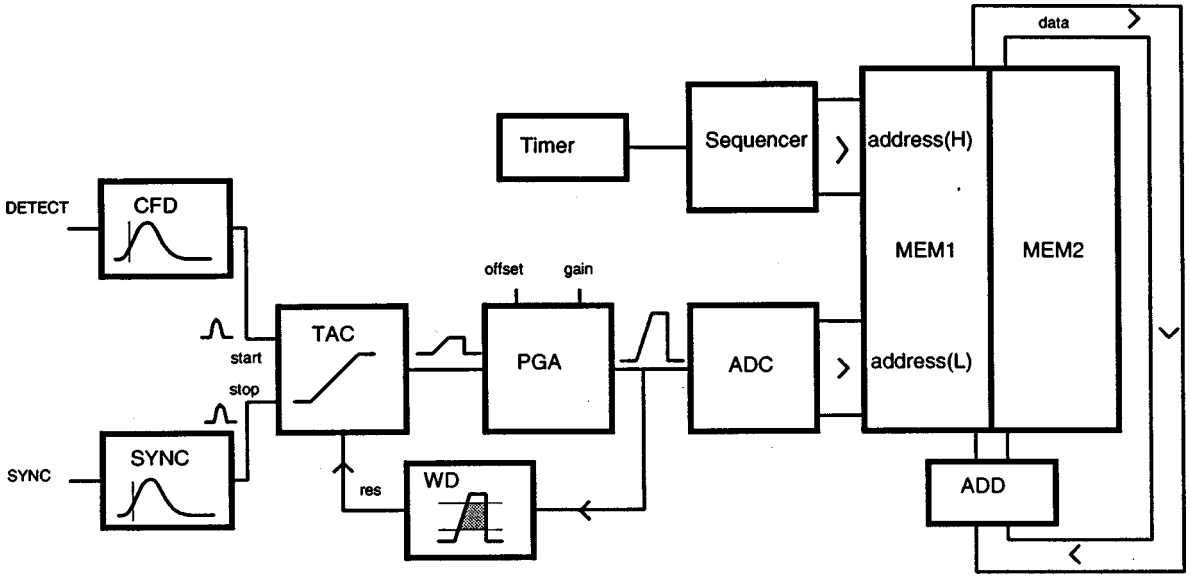

FIG. 2. Block diagram of the PC-interface card SPC-430.

small differential nonlinearity. Besides, the hybrid circuits reduce speed losses due to signal distortions on interconnection lines.

3. Analog-to-digital converter

The ADC converts the amplified TAC signal into a memory address (MEM). As in all TCSPC systems, the ADC must work with an extremely high accuracy. The ADC has a resolution from 64 to 4096 time channels and the width of each particular channel has to be constant within 1%–2%. This requires a ''no missing code'' accuracy of more than 18 bits which is not achievable with conventional fast ADCs which are, however, required to achieve a high count rate. In the SPC-430 module, the problem is solved by the use of a fast flash ADC with 12 bit no missing code accuracy in combination with a proprietary error correction method. The basic idea of this method is the implementation of a variable offset on the TAC characteristics referred to the ADC characteristics (Fig. 3). Thus, each photons event is converted at a slightly different position of the ADC characteristics resulting in an averaging of the errors of the ADC characteristics and a considerable reduction of the difference of the particular ADC steps. The arrangement is shown in Fig. 3. The digital-to-analog converter (DAC) is used to shift the TAC output voltage up and down on the ADC characteristics and is controlled by a counter which counts the start pulses of the TAC. Consequently, the DAC generates a sawtooth that increases by one DAC step at each recording of a photon. The DAC voltage is added to the TAC output voltage and the resulting signal is converted by the ADC. To restore the correct address byte for the memory, the counter bits are subtracted from the ADC result in a digital subtraction circuit. Of course, each address byte still contains an unavoidable deviation of the particular ADC step from the correct value. However, there is a significant difference to a direct ADC conversion in that the error is now different for different photons, even if the photons appeared at equal times and caused equal TAC voltages. Hence, the averaging of the individual errors results in a smoothing of the effective ADC characteristics.

The obtained improvement of the conversion accuracy depends on the number of ADC steps N_{ADC} over which the signal is shifted by the DAC voltage. Additionally, the accuracy depends on the distribution of errors of the ADC char-

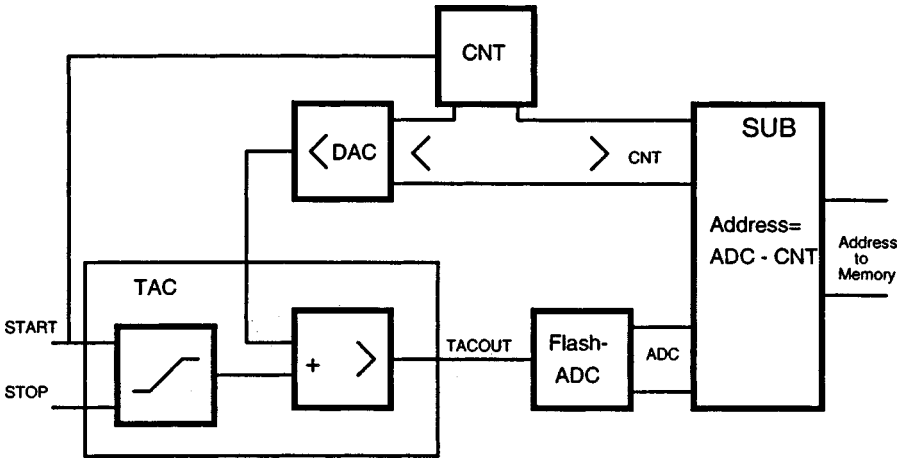

FIG. 3. A/D conversion of the SPC-430 with error correction.

TABLE I. Number of decay curves in the memory banks and minimum collection times per decay curve for gap-free measurements using a Pentium 200 processor.

ADC resolution (Number of channels)	Number of curves in one memory bank	Minimum collection time/curve without gaps (ms)
64	2048	0.15
256	512	0.60
1024	128	2.40
4096	32	9.60

acteristics. If the error of an ADC step is not correlated to the errors of adjacent steps the improvement is $(N_{\mathrm{ADC}})^{1/2}$. However, in real flash ADCs the errors appear periodically with periods of 4, 8, and 16 low significant byte. If a DAC amplitude is used which corresponds to a multiple of this value, the improvement in accuracy is considerably higher than $(N_{\mathrm{ADC}})^{1/2}$.

4. Memory control

Differing from conventional TCSPC devices, the data memory in the SPC-430 module consists of two memory banks with 128 k words, respectively. Depending on the selected ADC resolution (64, 256, 1024, or 4096 time channels) in each memory bank 2048, 512, 128, or 32 data blocks, i.e., decay curves, can be stored. Within a selected memory bank and data block, respectively, the buildup of the histogram is done by the usual method. When a photon is detected, the contents of the addressed location of the MEM is increased by a fixed increment. This is done by the ADD circuit. Values greater than 1 can be added to get full scale recordings in short collection times, e.g., for oscilloscope or single-molecule applications.

For single-molecule detection and similar applications a timer controlled sequencer logic is provided which automatically switches through all available data blocks. When the current memory bank has been filled with decay curves, the measurement continues in the other memory bank. While the measurement runs in one memory bank, the data from the other bank were read out and written to the hard disk of the computer. As long as the data stream is fast enough to read one complete memory bank before the other one is filled, subsequent decay curves can be measured without gaps between the curves. The minimum collection (integration) times per decay curve for a gap-free measurement for a Pentium 200 processor are given in Table I.

Multichannel-scalar (MCS) traces were generated by adding up all photons collected per integration time in an applied time window.

III. RESULTS AND DISCUSSION

Figure 4 shows a section of a data block derived by time-resolved fluorescence measurement of a 10^{-11} M aqueous solution (20 mM aqueous Tris-borate buffer *p*H 8.4, 30% glycerol, 5% Tween 20) Cy5-dCTP in an open volume. The integration time was set to be 1 ms per decay. The measurement was performed with an ADC resolution of 64 channels à 0.25 ns allowing 2048 decay curves to be collected in one

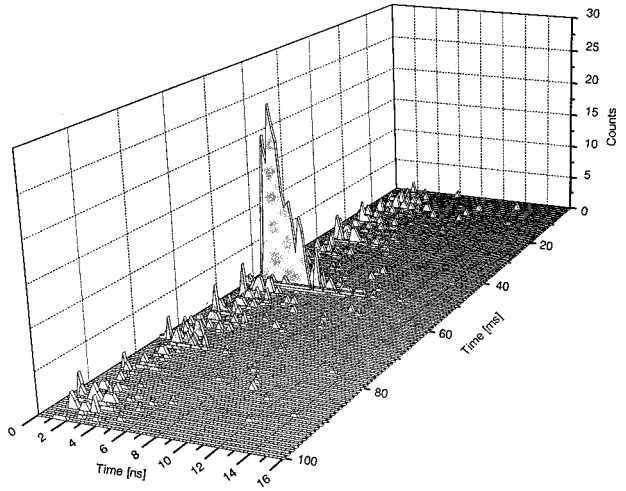

FIG. 4. Raw TCSPC fluorescence data recorded with the plug-in card SPC-430 from a 10^{-11} M aqueous solution of Cy5-dCTP (65% 20 mM Tris-borate buffer *p*H 8.4, 30% glycerol, 5% Tween 20) using a collection time of 1 ms per decay curve. Excitation power: 630 μW at the sample, ADC resolution: 64 channels à 0.25 ns. Approximately 50 ms after the start of the experiment a Cy5-dCTP molecule passes the detection volume and a decay curve containing about 200 fluorescence photons is received within 1 ms in the applied time-window of 16 ns.

memory bank (Table I). As can be seen, at approximately 50 ms after start of the experiment, a single Cy5-dCTP molecule passes the detection volume and about 200 photons are detected within 1 ms. With the chosen channel width, 25 photons were collected in the maximum channel by using a fluorescent dye (Cy5) with a relatively short fluorescence decay time in this solvent of 1.3 ns.[24] In Fig. 5, MCS-traces (3 ms per decay curve) are formed from the recorded data measured at the same sample. Figure 5(a) shows the MCS trace observed from the same sample in an open volume. In Figs. 5(b)–5(f), the sample is measured flowing through the thin end in the microcapillary at different applied voltages. With the setup described, an average background of 2 kHz was obtained in the solvent system at hand in an open volume. This background arises mainly from Raman scattered photons passing the emission filter simultaneously with the excitation pulse. The same background rate was obtained in the microcapillary at the thin end of the tip. On the basis of this background, we calculate signal-to-background ratios (S/B) of up to 200 for the most intense peaks. It should be noted that in case of an open volume the analyte molecules move in and out of the detection volume due to their Brownian motion. Hence, most analyte molecules will cross the detection volume at the edge resulting in small burst sizes. However, if the detection took place inside of a microcapillary with an inner diameter smaller than the detection area of the optical system, all analyte molecules should be excited and detected very efficiently. In addition. Figs. 5(b)–5(f) demonstrates that a control of the analyte movement towards the anode is possible by applying different voltages. On the one hand, the number of analyte molecules passing the detection area increases with increasing voltage. Simultaneously, the velocities increase thereby decreasing the burst size.

To study the burst size, the fluorescence decay time, and

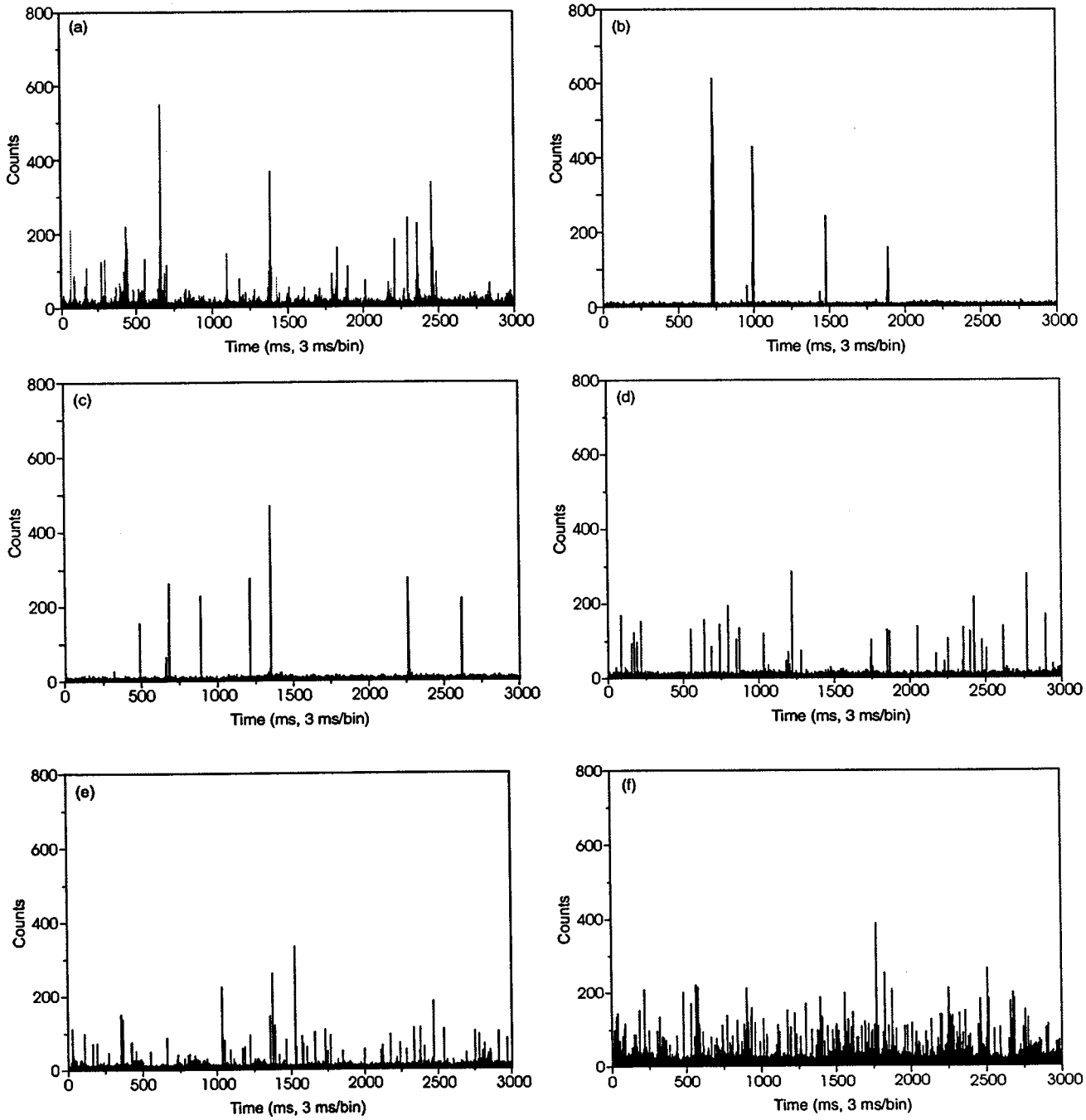

FIG. 5. Fluorescence signals observed from a 10^{-11} M solution of Cy5-dCTP in 65% 20 mM Tris-borate buffer *p*H 8.4, 30% glycerol, 5% Tween 20 with an integration time of 3 ms per bin. the data were collected with the PC-module SPC-430 for TCSPC and converted into MCS traces. (a) Open volume, (b), (c), (d), (e), and (f) microcapillary at different applied voltages of 2, 5, 9, 15, and 30 V, respectively.

the burst length of each individual burst, we used a burst recognition procedure. To suppress the background (2 kHz) efficiently, only bursts with a count rate higher than 10 kHz were utilized. The starting and the end point of a burst were defined by a count rate of 5 kHz. For the case that two count rate maxima fall into the same time interval, we split the bursts at the minimum count rate between them. For the following statistics we used more than 4000 single-molecule events measured at different applied voltages, respectively. Figures 6(a)–6(c) show the histograms of the burst sizes, the fluorescence lifetimes, and the burst lengths of Cy5-dCTP molecules measured in the microcapillary at 5 V with an integration time of 500 μs/bin and an ADC resolution of 64 channels à 0.25 ns. As shown in Figs. 6(a) and 6(c), we determine an average burst size of 141 counts (maximum frequency 110 counts) and an average burst length of 5 ms (maximum frequency 3.5 ms) at an applied voltage of 5 V. Using this data, we calculate an average S/B of 14 (S/B=17 using the frequency maxima) for individual Cy5-dCTP measured in a microcapillary with an inner diameter of 500±200 nm. With a diameter of the detection area of 1 μm, we calculate a flow speed of about 0.3 mm/s for Cy5-dCTP molecules in the described solvent at a voltage of 5 V. The fluorescence decay time determination of the single-

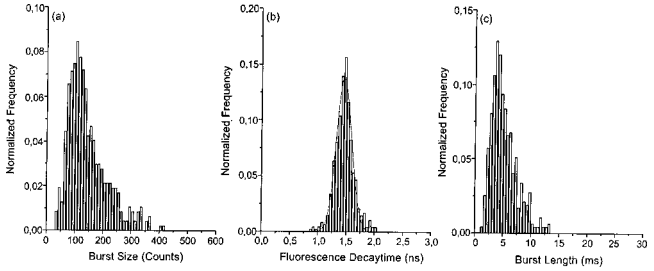

FIG. 6. (a) Burst size, (b) fluorescence decay time, and (c) burst length distribution measured on a 10^{-11} M solution of Cy5-dCTP flowing through the microcapillary at an applied voltage of 5 V. Data acquisition was performed with PC-module SPC-430 with an integration time of 0.5 ms per decay curve, ADC resolution of 64 channels, number of decay curves in one memory bank: 2048, 100 cycles, corresponding to a time period of 102.4 s without any loss of data.

molecule events was realized by a monoexponential maximum likelihood estimator (MLE) using the following relation:[26]

$$1+(e^{T/\tau}-1)^{-1}-m(e^{mT/\tau}-1)^{-1}=N^{-1}\sum_{i=1}^{m} iN_i, \qquad (1)$$

where T is the width of each channel, m the number of utilized time channels, N the number of photon counts taken into account, and N_i the number of photon counts in channel i. The parameters used to determine the decay time τ are $m=50$, $T=0.25$ ns. To calculate the experimental standard deviation σ_{exp} the obtained fluorescence decay times were plotted against their frequency [Fig. 6(b)]. Applying a Gaussian fit to the fluorescence decay time distribution results in a fluorescence decay time of $\tau=1.45$ ns with an experimental standard deviation $\sigma_{\text{exp}}=0.14$ ns, which is in good agreement with the fluorescence decay time of Cy5-dCTP measured in bulk solutions in the same solvent ($\tau_{\text{bulk}}=1.3$ ns).[24]

In Fig. 7(a), the number of detected bursts per second observed from a 10^{-11} M Cy5-dCTP solution in a microcapillary at different applied voltages are shown. As expected from Fig. 5 with increasing voltage the number of detected analyte molecules increases linear up to an applied voltage of 30 V. In addition, also the velocity of the analyte molecules through the detection volume with a diameter of 1 μm is proportional to the voltage [Fig. 7(b)]. Since in an open volume most analyte molecules pass at the edge of the detection volume, the measured average diffusion time of 0.9 ms[24] (corresponding to a velocity of 1.1 mm/s) is shorter than the average value obtained from analyte molecules passing the detection volume in the microcapillary due to electrokinetic forces. In the microcapillary, all analyte molecules have to cross at the center of the detection volume with a diameter of 1 μm. This indicates that we deal essentially with a 1-dimensional diffusion, i.e., our detection volume perpendicular to the flow direction is not defined by the confocal system but by the capillary walls. Hence, all analyte molecules cross the center of the detection area with a diameter of 1 μm, i.e., the measured average diffusion time is longer (corresponding to a slower velocity) than in open volumes.

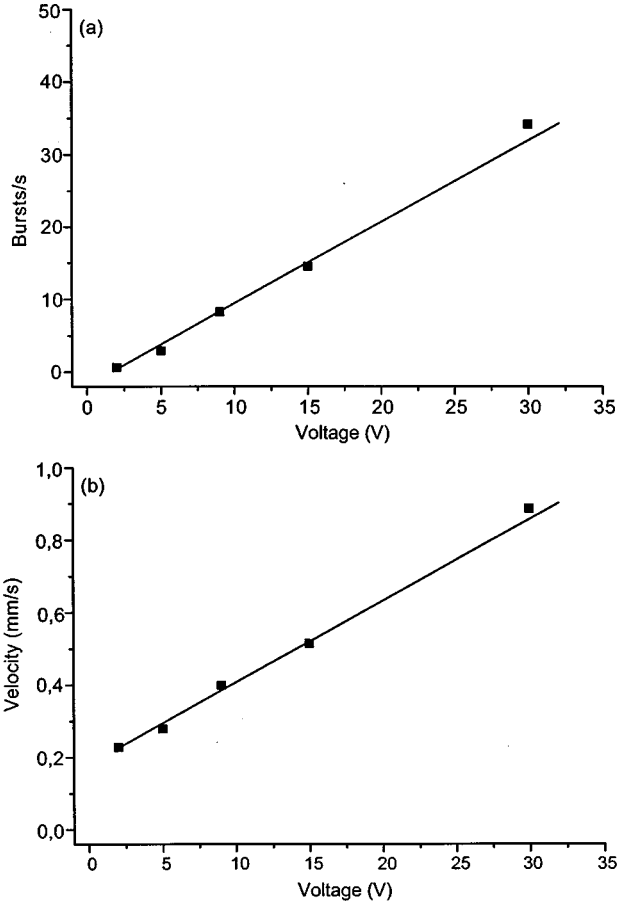

FIG. 7. (a) Detected bursts per second, and (b) velocities of Cy5-dCTP molecules (10^{-11} M) measured in the microcapillary with a collection time of 0.5 ms pr decay curve at different applied voltages. The velocity of the analyte molecules through the detection volume was calculated from the maximum frequency obtained from the burst length distribution shown in Fig. 6(b) and a diameter of the detection area of 1 μm.

IV. DISCUSSION

Counting and time-resolved identification of individual labeled analyte molecules in a microcapillary with an inner diameter of 500 ± 200 nm have been demonstrated. As shown, the characteristic flow velocity of negatively charged analyte molecules in the capillary is proportional to the applied electrical field and is slower than the velocity measured in an open volume element.[24] This behavior can be addressed to 1-dimensional diffusion characteristics indicating that the adsorption of the analyte molecules on the glass surface can be neglected by addition of Tween 20. Furthermore, the electroosmotic flow is drastically reduced allowing a control of the motion of negatively charged molecules towards the anode. By the use of a new PC plug-in card (SPC-430) the fluorescence decay times, burst sizes, and burst lengths, i.e., diffusion times, can be measured for each individual analyte molecule present in a given sample. The results presented demonstrate new possibilities for time-resolved single strand DNA sequencing[9] and other important bioanalytical applications such as highly sensitive detection of ribonucleic acid, DNA sequences or proteins due to different electrophoretic mobilities of bound and free labeled primers and antibodies, respectively, in the microcapillary.

ACKNOWLEDGMENTS

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January 2002

TCSPC Laser Scanning Microscopy

Upgrading laser scanning microscopes with the SPC-730 TCSPC lifetime imaging module

Fluorescence Lifetime Imaging (FLIM) has become a new powerful method to investigate molecular interactions, metabolic reactions and fluorescence quenching or energy transfer in cells and subcellular structures [4-7]. These effects cause changes in the fluorescence quantum efficiency and thus in the fluorescence lifetime. Since the fluorescence lifetime does not depend on the unknown dye concentration it is a direct measure for the quantum efficiency. It therefore gives a more direct access to the investigated effects than the fluorescence intensity. Furthermore, the fluorescence lifetime can be used to separate the fluorescence of different luminophores in the cells if the components cannot be distinguished by their fluorescence spectra.

Recording time-resolved fluorescence images can be achieved by combining a Laser Scanning Microscope with pulsed laser excitation and a new Time-Correlated Single Photon Counting (TCSPC) Imaging technique introduced by Becker & Hickl [1, 6]. This note was written to assist upgrading of laser scanning microscopes for lifetime imaging.

Introduction

The Light Source

Fluorescence Lifetime Imaging requires a pulsed excitation source with a repetition rate in the MHz range.

Titanium-Sapphire Lasers

The ultimate solution is the femtosecond Ti:Sa laser. These lasers deliver pulses with 70 to 80 MHz repetition rate, 80 to 200 fs pulse width and up to 1 W average power. The wavelength is in the NIR from 780 nm to 950 nm. Tuneable and fixed wavelength versions are available. To excite the sample which usually absorbs below 500 nm, simultaneous two photon excitation is used. Due to the short pulse width and the high energy density in the focus of the microscope the two-photon process works very efficiently. Therefore the traditional frequency doubling of the Ti:Sa radiation is not normally used for laser scanning microscopes.

Frequency Doubled Titanium-Sapphire Lasers

Frequency doubled titanium-sapphire lasers can be used to excite the sample via the traditional one-photon absorption. Frequency doubling is achieved by a nonlinear crystal. The output power is in the mW range. Less than 50µW are required to excite a typical sample so that the available power is by far sufficient. Whether one-photon or two-photon excitation gives less photodamage is still under discussion. In a few cases we obtained considerably higher count rates and less photodamage for one-photon exciation.

If a frequency doubled Ti:Sa laser is used, the NUV pulses should be stretched to a width of some ps. This avoids two-photon processes into the UV which are absolutely deadly for any biological sample.

Fibre Lasers

Another useful excitation source are fibre lasers. Fibre lasers are available for a wavelength of 780 nm and deliver pulses as short as 100 to 180 fs [8]. The average power is 10 to 20 mW. This is less than for the Ti:Sa laser but well sufficient for two-photon excitation. As a rule of thumb, the maximum useful power for biological samples and fs NIR excitation is 1 to 100 mW. A higher power kills the cells or cooks the sample. The benefit of the fibre laser is the small size, the high reliability and the lower price compared to the Ti:Sa laser. The drawback is the fixed wavelength.

Pulsed Diode Lasers

A reasonable cost solution for one-photon excitation are pulsed diode lasers which are available for the blue and red spectral range [9]. These lasers deliver pulses from 40 to 400 ps duration with up to 80 MHz repetition rate. The average power is a few mW. The beam quality of diode lasers is not very good. Therefore it can be difficult to obtain a diffraction-limited resolution. However, if only the central part of the beam is used, the result can be quite acceptable. Discarding a large fraction of the beam causes a considerable loss of power. This loss is, however, not substantial because 50 µW are absolutely sufficient to excite the sample.

The microscope companies sometimes suggest to use a continuous laser and to modulate it with an electro-optical modulator. Don't use this solution. It is unable to deliver sufficiently short pulses and more expensive than a good diode laser.

The Microscope

The general optical principle of a laser scanning microscope is shown in fig. 1.

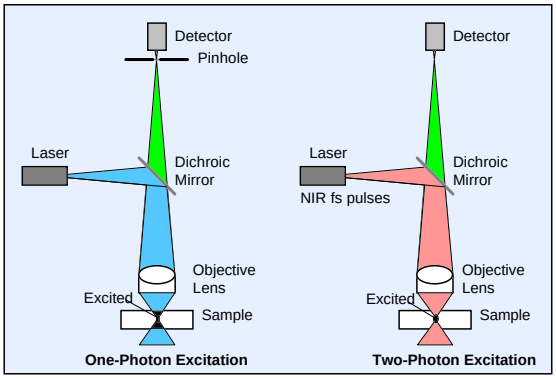


Fig. 1: Principle of a laser scanning microscope (scanning setup not shown)

The laser is fed into the optical path via a dichroic mirror and focused into the sample by the microscope objective lens. In the traditional one-photon setup the light from the sample goes back through the objective lens, through the dichroic mirror and through a pinhole in the upper image plane of the objective lens. Light from outside the focal plane is not focused into the pinhole plane and therefore substantially suppressed. X-Y imaging is achieved by optically scanning the laser spot over the sample, Z imaging (optical sectioning) is possible by moving the sample or the microscope up and down.

With a fs Ti:Sa laser the sample can be excited by two-photon absorption. Due to the short pulse duration and the small diameter of the Airy disk the photon density in the focus is very high, so that the two-photon excitation works with high efficiency. Excitation occurs only in the focus, so that no pinhole is required to reject light from outside the focal plane. For imaging biological samples, two-photon excitation can have several benefits:

- Scattering and absorption coefficients at the near infrared excitation wavelength are small. Therefore, layers deeply in the sample can be excited [4].
- If deep sample layers are imaged the scattering at the emission wavelength broadens the luminescent spot seen by the microscope objective. The absence of a pinhole allows to efficiently record the emitted light from a relatively large area while maintaining the optical sectioning capability.
- Living cells are surprisingly stable under two-photon NIR excitation [7]. The reason is that the absorbed energy is far less than the energy in the excitation beam. Furthermore, there is no substantial absorption above and below the focal plane.

The TCSPC Imaging Module

The principle the SPC-730 TCSPC Imaging module [1] is shown in fig. 2.

The module employs an advanced TCSPC technique featuring both high count rate and low differential nonlinearity [1]. It contains the usual building blocks (CFDs, TAC, ADC) in the 'reversed start-stop' configuration together with a scanning interface and a large histogram memory integrated on one board.

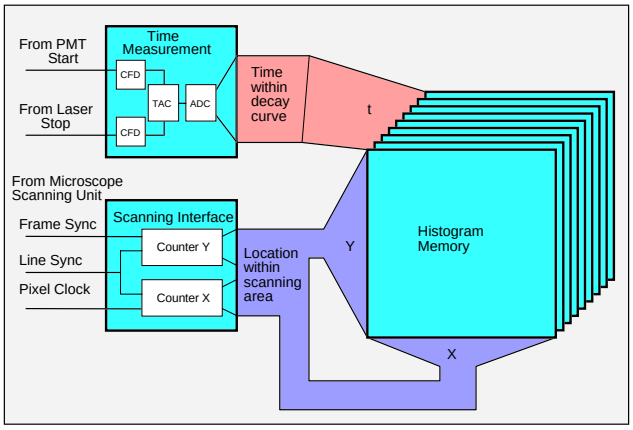


Fig. 1: Basic principle of the SPC-730 TCSPC Imaging module [1]

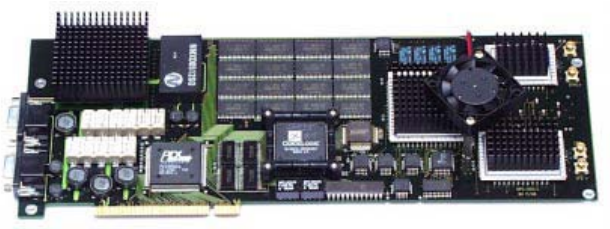


Fig. 2: The Becker & Hickl SPC-730 TCSPC Imaging Module [1]

The TCSPC module receives the single photon pulses from the photomultiplier (PMT) of the microscope, the reference pulses from the laser and the Frame Sync, Line Sync and Pixel Clock signals from the scanning unit of the microscope. For each PMT pulse, i.e. for each photon, the TCSPC module determines the time of the photon within the laser pulse sequence and the location within the scanning area. These values are used to address the histogram memory in which the events are accumulated. Thus, in the memory the distribution of the photon density over x, y, and the time within the fluorescence decay function builds up. The result can be interpreted as a two-dimensional (x, y) array of fluorescence decay curves or as a sequence of fluorescence images for different times (t) after the excitation pulse.

Interestingly, there is practically no loss of photons in the TCSPC imaging process. As long as the photon detection rate is not too high all detected photons are processed and accumulated in the histogram, thus providing maximum sensitivity. This is a key advantage of the TCSPC Scanning Microscope over simple (single gate) gated photon counting or gated image intensifiers which gate away the majority of the fluorescence photons.

Another benefit of TCSPC imaging is the high time resolution. The time channel width can be as small as 820 fs. Decay times down to 5 ps can be determined with fast detectors. On the

other hand, TCSPC imaging is flexible in that a large number of pixels can be measured with wide time channels or precision measurements can be done at a small number of pixels. Furthermore, the method can work with the fastest scanning speed of the microscope thus avoiding heat concentration in the excited spot of the sample.

Advocates of gated photon counting or phase fluorometry sometimes claim that the count rate of the TCSPC method is too low for microscopy application. This ill reputation comes from older TCSPC devices built from nuclear instrumentation modules. The SPC-730 has a dead time of 180 ns yielding a maximum useful count rate of about 3 MHz. For comparison, living cells excited by two-photon excitation give a count rate of some 10,000 photons per second. Samples of non-living cells stained with highly fluorescent dyes can deliver up to 200,000 photons per second. A higher excitation intensity kills the cells or destroys the sample.

The Detector

Time-correlated single photon counting requires a detector capable to deliver an electrical pulse for a single detected photon. The detector must be fast enough to resolve the fluorescence decay time of typically 100 ps to 5 ns. Only photomultipliers (PMTs) and single-photon avalanche photodiode modules meet these requirements.

Built-in PMTs

Commercial scanning microscopes employ small side-window PMTs to detect the light from the sample. The PMTs are integrated in the scanning head. Usually there are several detection channels with separate PMTs. The PMTs are selected for optimum sensitivity, not for time resolution in the single photon mode. Thus, the built-in detectors can be used for TCSPC imaging, but do not deliver a good time resolution. Nevertheless, they can be used to distinguish between different dyes in multi-stained samples and to investigate other lifetime effects in the ns range. They are **not** useful for FRET measurements. The internal detectors should be operated at the maximum permissible supply voltage and with a HFAC26-10 preamplifier of Becker & Hickl.

PMH-100 PMT Module

The PMH-100 of Becker & Hickl is a rugged PMT module with an internal high-voltage generator, preamplifier, and overload warning circuit. It is connected directly to the SPC-730 module. The PMH-100 delivers a system response of 150 to 220 ps FWHM and can be used to measure lifetimes down to 200 to 300 ps. We recommend this detector as a startup solution. It is attached to the non-descanned port of the microscope or to the a fibre output from the scanning head. The PMH-100 is available with bialkali and multialkali cathodes. The reduced spectral range of the bialkali cathode can be a benefit in two-photon systems when blocking of the laser line is a problem. Typical FRET effects are well detectable with the PMH-100. However, for quantitative FRET experiments we recommend the R3809U MCP-PMT (see below).

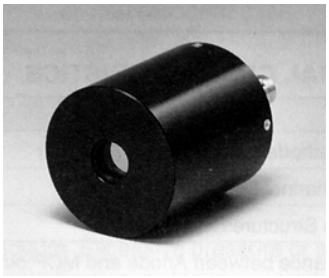
PMH-100, about 1/2 natural size

H5783 Photosensor Module

H5783 module, natural size

The Hamamatsu H5783P and H5773P Photosensor Modules incorporate a small size PMT and the HV power supply. They require a +12 V supply and some gain setting resistors only. The +12 V is available from the SPC-730 module or from the DCC-100 detector controller (see below). The time resolution is 150 to 220 ps FWHM. The H5783P and H5773P are available with bialkali and multialkali cathodes. For optimum results, use the '-P' type, which is specified for photon counting. Due to their small size, the H5783 and H7783 modules are a solution if a detector in the scanning head has to be replaced with a faster one. The modules should be operated with a HFAC-26-10 preamplifier of Becker & Hickl.

R3809U MCP PMT


R3809U MCP, about 2/3 natural size

The Hamamatsu R3809U MCP PMT [2, 3] achieves an FWHM below 30 ps. It is the ultimate solution for TCSPC scanning microscopes. It is clearly the best detector for FRET experiments. However, since MCPs are expensive and easily damaged it is neither a solution for beginners nor a low budget solution.

The R3809U is connected to the SPC-730 via an HFAC-26-01 preamplifier. Furthermore, it requires a 3 kV high voltage power supply. Thus, the overall cost for one R3809U detection channel is in the order of $15,000 to $20,000. This is not very much compared to the price to the microscope and the laser, but a lot if the detector is damaged by maltreatment. Due to its relatively large size the R3809U should be attached to the non-descanned port of the microscope or to a fibre output from the scanning head.

The R3809U is available in different cathode versions. The most versatile one is the R3809U-50 with a multialkali cathode for the wavelength range from 180 to 820 nm. The R3809U-52 has a bialkali cathode and can be used up to 650m [3]. This limitation of the spectral range can be a benefit for two-photon systems when blocking of the laser line is a problem.

Gain control and overload shutdown of the R3809U can be achieved by the DCC-100 detector controller, see below.

A cooler is available for the R3809U. Cooling substantially reduces the dark count rate and therefore makes possible long acquisition times. However, before you install a cooler, make sure that your background signal really comes from the detector and not from poor blocking of excitation light or even from insufficient shielding of daylight.

SPCM-AQR Avalanche Photodiode Modules

The SPCM-AQR Avalanche Photodiodes Modules of EG&G / Perkin Elmer have a high quantum efficiency in the NIR. This makes the modules exceptionally suitable for single molecule investigations. For precision decay time measurements they are less useful because the system response is 500 ps wide and slightly dependent on the count rate. The SPCM-AQR is connected to the SPC-730 via an adapter available from Becker & Hickl.

Hamamatsu H7222 Modules

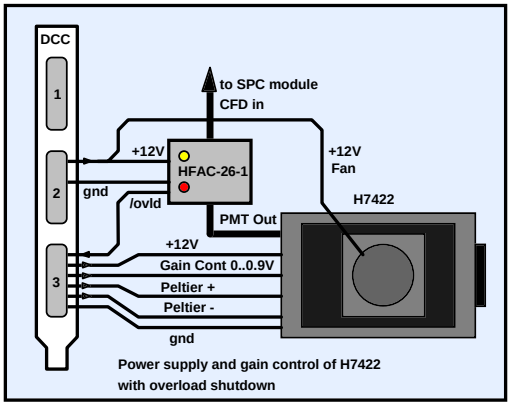
The H7422 modules are a high speed, high sensitivity PMT modules. They contain a GaAs photomultiplier along with a thermoelectric cooler and a high voltage generator. The H7422 modules feature excellent sensitivity in the red and near-infrared region. The resolution in the TCSPC mode is typically 250 ps. The H7422 comes in different cathode versions for the wavelength range up to 900 nm. For most microscope applications the H7422-40 is best. It has the highest quantum efficiency of all H7422 versions and is sensitive up to 750 nm. Above this wavelength the sensitivity drops rapidly. That means that the dyes typically used for cell staining can be measured, but there is a substantial suppression of the excitation line of a Ti:Sa laser for two-photon excitation.

If you need sensitivity up to 900 nm - which requires one photon excitation in the red or NIR range - you can use the H7422-50.

All H7422 modules must be handled with care because the cathodes can easily be damaged by overload. Exposure to daylight is not allowed even when the devices are switched off. Therefore, the H7422 should be used with an HFAC-26-1 preamplifier only. Gain control, overload shutdown and cooling can be achieved by using the **bh** DCC-100 detector controller (see below).

The DCC-100 detector controller

The DCC-100 module is designed to control detectors in conjunction with **bh** photon counters. It can be used to control the gain of the Hamamatsu H7422, H5783, H6783, H7422 or similar Hamamatsu photosensor modules by software. The gain of MCPs and PMTs can be controlled via the FuG HCN-14 High Voltage Power Supply. In conjunction with **bh** preamplifiers, overload shutdown of the detectors can be achieved. Furthermore, the DCC-100 delivers the current for thermoelectric coolers, e.g. for the Hamamatsu H7422. High current digital outputs are available for shutter or filter control. The DCC-100 is a PCI module for IBM compatible computers. It works under Windows 95, 98, 2000 and NT.



The figure right shows how a H7422 module is controlled via the DCC-100. For more information, please see DCC-100 data sheet and DCC-100 manual, www.becker-hickl.com.

Preamplifiers

Most MCPs and PMTs deliver pulses of 20 to 50 mV when operated at maximum gain. Although these pulses can easily be detected by the input discriminators of the SPC modules a preamplifier can improve the time resolution, the noise immunity, the threshold accuracy and the safety against damaging the SPC input. Furthermore, it can extend the detector lifetime because the detector can be operated at a lower gain and a lower average output current.

For TCSPC applications we recommend our HFAC-26 preamplifier. The HFAC-26 has 20 dB gain and 1.6 GHz bandwidth. The maximum linear output voltage is 1 V. Therefore, it amplifies the single photon pulses of a typical PMT or MCP without appreciable distortions. Furthermore, the HFAC-26 incorporates a detector overload detection circuit. This circuit measures the average output current of the PMT and turns on a LED and activates a TTL signal when the maximum safe detector current is exceeded.

HFAC-26 Amplifier

Thus, even if the gain of the amplifier is not absolutely required the overload warning function helps you to make your measurement setup ‘physicist proof’. If you use an MCP with your SPC module you should always connect it via an HFAC-26 preamplifier.

The HFAC-26 is available with different overload warning thresholds from 100 nA (for MCPs) to 100 uA (for large PMTs).

System Setup

Attaching the detector

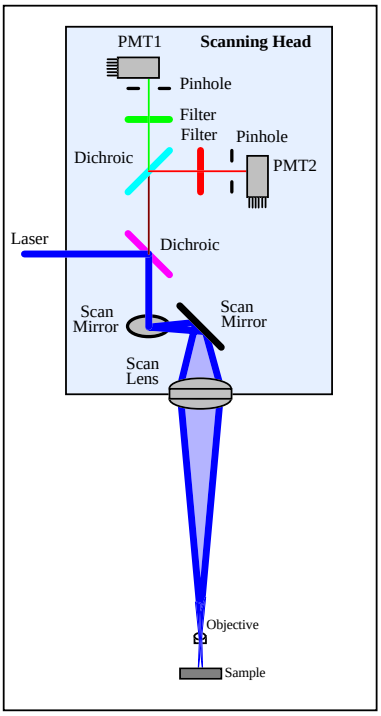
Generally there are four options for the detection channel. You can

- use one of the internal detectors of the microscope
- replace one of the internal detectors with a faster one
- use a fibre to feed the light from the scanning head to an external detector
- attach a detector to the non-descanned port of the microscope

Not all options are available in all microscopes. The following considerations can help to find the best solution for your microscope and your application.

Using the built-in detectors

The general principle of the scanning head of a laser scanning microscope is shown in the figure right.



The laser beam is coupled into the setup via a dichroic mirror. Than it is deflected by the scan mirrors. The scan lens focuses an image of the scan mirror axis into the microscope objective. Thus, the direction the laser takes through the objective changes as the mirrors tilt back and forth thus scanning the laser focus over the sample. The fluorescence light from the sample goes back through the microscope objective, the scan lens, and the scan mirrors. If the setup is correctly aligned the motion of the returned light beam is exactly compensated by the scan mirrors. The fluorescence light is separated into several detection channels by one or several additional dichroics. Filters are used to block the scattered laser light and to select the correct fluorescence wavelength. Pinholes in front of the detectors are used to suppress the light from outside the focal plane of the microscope objective.

Various modifications of this setup are used in different microscopes. Transfer lenses or mirrors are used to image the rotation axis of the first scanning mirror into the axis of the second one, the laser beam diameter is changed to fit the diameter of the microscope objective, monochromators are used instead of the filters, and the size of the pinholes can be adjustable. Depending on the application and on the setup of the scanning head, detection via the scanning head PMTs has benefits and drawbacks.

- One-photon excitation requires the pinholes to suppress the fluorescence from outside the focal plane. Therefore, the detection path back through the scanning head is the only useful one for one-photon excitation.

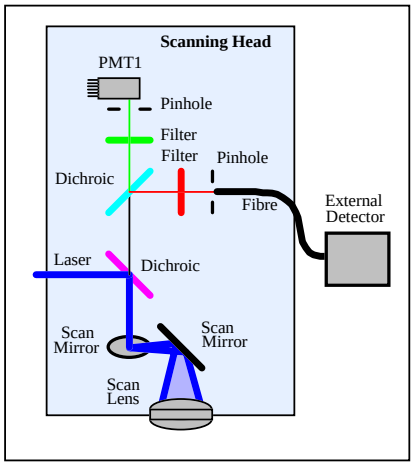
- Two-photon excitation does not require the pinholes. The pinholes can even be troublesome because they suppress some light that leaves the sample slightly scattered. Therefore, the pinhole size is adjustable in good scanning heads. If the pinholes can be made wide enough, there is no appreciable drawback of the detection path via the scanning head.

- For two-photon excitation, the complicated optical path trough the scanning head can introduce some loss of intensity due to the large number of lens and mirror surfaces. However, the scanning heads of state-of-the-art microscopes are optically near perfect so

that there is no noticiable loss of photons. Furthermore, computer controlled selection of dichroics and filters or monochromators available in the scanning heads helps to select the best wavelength range and to suppress scattered laser light. This often compensates for possible loss in the optical path.

- The pinhole - even a very large one - helps to suppress straylight and optical reflections that often show up in time resolved data obtained by non-descanned detection.
- The most serious drawback of using the internal detectors is the poor time resolution. Unfortunately, replacing the detectors with faster ones is usually very difficult, if not impossible. The remedy is the fibre coupling option described in the next section.

Fibre Coupling



One of the internal detectors is removed and an optical fibre is attached instead. The light is fed to an external detector. Compared to the setup with all PMTs in the scanning head, this configuration has the benefit that any detector can be installed at the end of the fibre. There is no problem to use large detectors, coolers, or additional filters.

The drawback is that

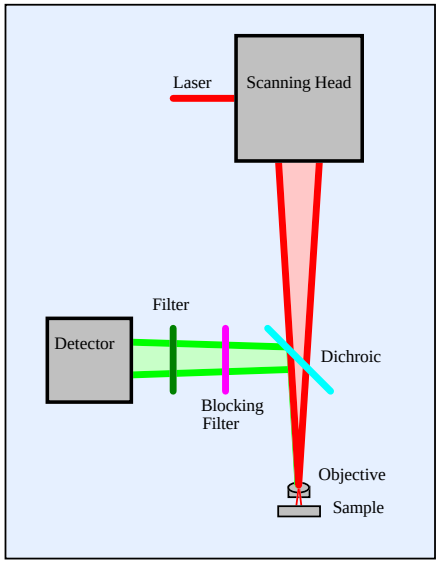
- the coupling of the fluorescence light into the fibre can be inefficient, especially for a thin fibre in conjunction with two photon excitation and strongly scattering samples.
- the dispersion in the fibre can introduce some broadening of the system response. Due to the small aperture of the light beam behind the pinhole the effect is very small. We did not find a substantial loss of resolution of an R3809U MCP coupled trough a 1 m long fibre to a Zeiss LSM-510.

Some microscope manufacturers (e.g. Zeiss) offer a fibre coupling option for their scanning heads. In this case fibre coupling is an excellent and easy-to-use solution that works for one-photon and two-photon excitation as well. Detectors for the Zeiss LSM-510 fibre output are available from **bh**, see figure below.

PMH-100 H7422-40 R3809U
Detector for the 'Fibre Out' version of the Zeiss LSM-510

Non-Descanned Detection

Microscope manufacturers claim that non-descanned detection (NDD) is the ultimate solution for two-photon excitation. The optical path is shown in the figure right.



The fluorescence light is separated from the excitation by a dichroic mirror before it enters the scanning head. The light goes through a laser blocking filter and a wavelngthe selection filter directly to the detector. Although the principle is more or less the same in all microscopes, several modifications are possible. The setup of the dichroic can be reversed so that the scanned laser beam is reflected instead of the fluorescence signal. A demagnification lens can be used to shrink the illuminated area on the detector. Zeiss offers a 'Non Descanned Detection Module' with several detection channels and computer selectable dichroics and filters for the LSM-510.

The benefits and drawbacks of non-descanned detection are:

- Two-photon excitation in conjunction with NDD allows imaging as deep as 100 um into biologic tissue.
- For single cell layers there is no appreciable advantage in sensitivity compared to descanned detection. The scanning head optics in good microscopes works virtually without losses. If the pinhole diameter is adjustable (as it is in the Zeiss LSM-510) the advantage of NDD is questionable.
- NDD can be reasonably used only for two-photon excitation
- The selection of the dichroic and of the laser blocking filter is crucial. The scattered laser light is many orders of magnitude stronger than the fluorescence, and a suppression factor of 10^6 to 10^{10} is required. Therefore, make sure that the correct filters be inserted in your microscope and that your microscope supplier gives you appropriate support.
- Since there is no pinhole the NDD setup is prone to optical reflections. Reflections between the filters or reflections from condensor lenses behind the sample are often found in the decay curves.
- Since the detection path is relatively open to straylight the detector can easily be overloaded. Furthermore, NDD setups are often not safe in terms of operator errors. Often a mercury or halogen lamps used to adjust the sample visually. If the lamp can be switched on when the detection path is open the detector is immediately destroyed. Therefore, special care has to be taken in order not to damage the detector.

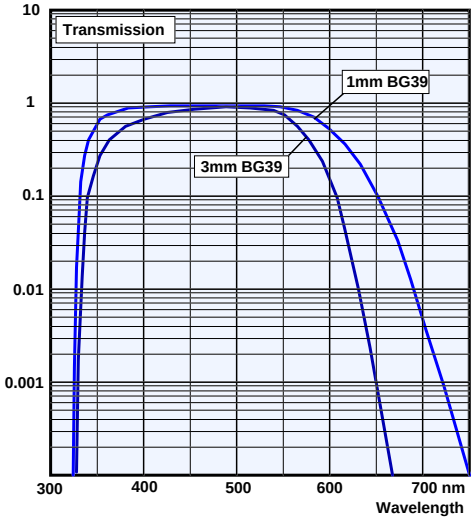
Two-Photon Systems

Optical System

Scattering of excitation light can be a serious problem in a two-photon microscope. Even if the optical system is perfect the excitation light scattered at the dichroic, at the microscope lens, and in the sample is many orders of magnitude stronger than the fluorescence light.

Therefore proper blocking of the excitation light is essential for two-photon fluorescence imaging.

The key to two-photon operation is the Schott BG39 filter. The characteristics of this filter is shown in the figure right. The filter efficiently blocks the excitation between 780 and 900 nm. A minimum of 1mm BG39 for bialkali detectors and a minimum of 3mm for multialkali detectors is required.



Transmission of BG39 Filter

Although dielectric filters have a sharper edge than the BG39 these filters usually fail to block the laser sufficiently. The reason may be that a dielectric filter reflects the light instead to absorb it. Therefore the laser light is scattered through the microscope and eventually arrives at the detector. Therefore, a dielectric filter should always be used in conjunction with a BG39.

Even with the BG39 filter scattered laser light often impairs the results of lifetime measurements. If the light comes from the sample it shows up as a sharp peak at the top of the fluorescence decay curves. If it comes from parts of the optical system the peak appears in a different position. If you see such effects although you have enough BG39 filters in the light path the reason may be:

Scattering at the microscope objective. To get diffraction limited resolution, the cross section of the laser beam is usually made larger than the microscope lens. A part of the laser beam hits the lens mount and is scattered into the detection path. Solution: Keep the beam diameter as small as possible - if you can. Or - if you are designing your own system - use a transfer lens and a diaphragm in front of the detector.

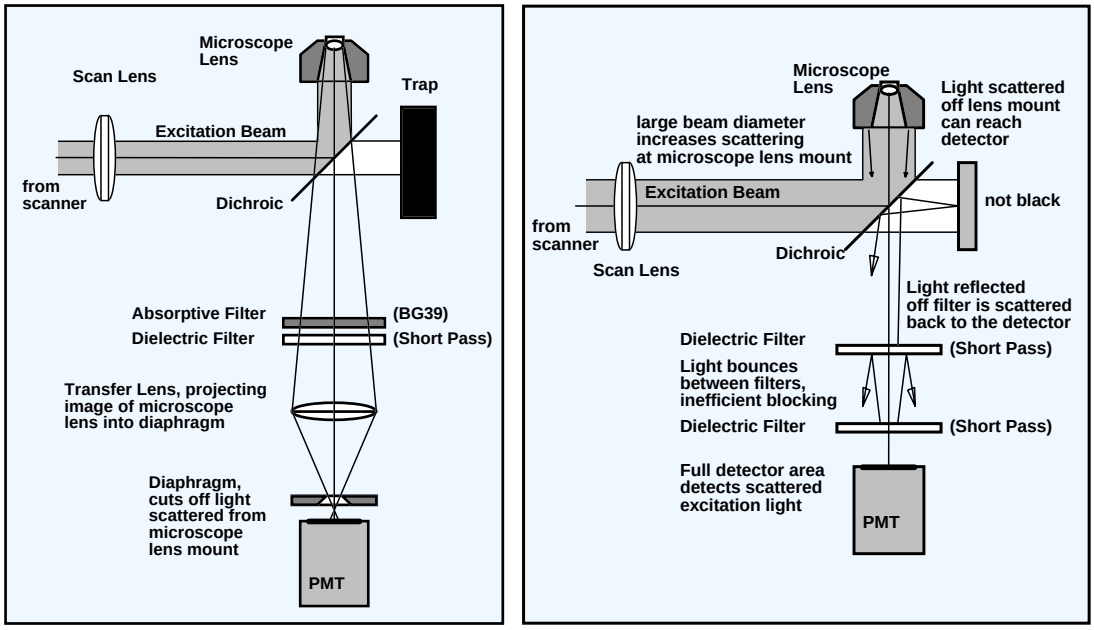
Reflection at a condensor lens. Usually the microscope has a condensor lens on the opposite side of the sample. Laser light that penetrates the sample can be reflected at this lens and directed back into the detection path. A simple solution is to shed the condensor during the measurement.

Dielectric blocking filters. In dielectric (dichroic) filters, the blocked light is reflected. That means, it is scattered through the microscope. Therefore, the first filter should be an absorptive filter, e.g. a BG 39.

Stacks of dielectric Filters. Do not stack dichroic filters to improve the blocking factor. The blocked light is reflected and bounces between the filters so that the blocking factor is less than the product of the blocking factors of the two filters. If dichroic filters have to be stacked, place absorptive filters (BG glass filters) between.

Insufficient Baffling: As far as possible, block straylight out of the detection path. The most critical places are the area around the microscope lens and the area behind the dichroic mirror. Excitation light from these areas can be diverted directly into the detection path. Make sure that the critical areas are black and insert baffles so that they are not directly seen by the detector.

A well-designed optical system for non-descanned detection and a design with features to be more or less avoided are shown in the figure below.



Well-designed NDD system (left) and features to be avoided (right)

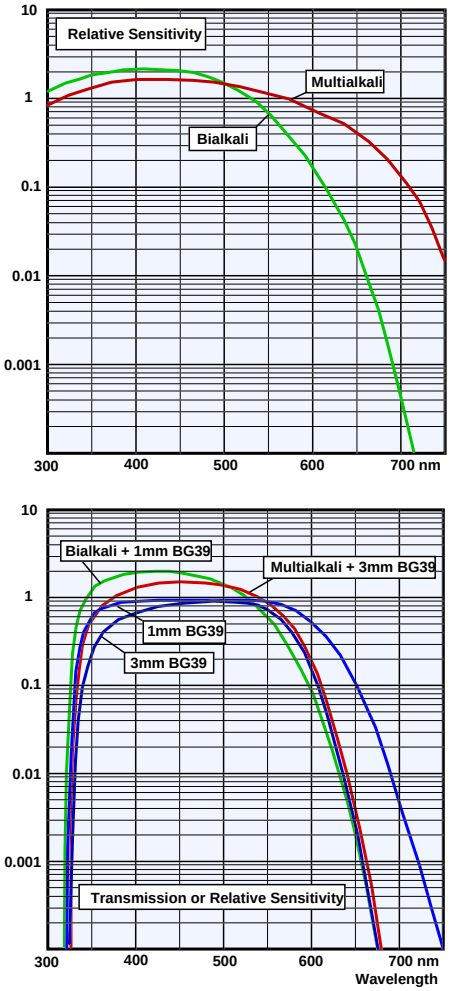
Selection of Detectors

For most detectors two cathode versions exist - the bialakali and the multialkali cathode. The quantum efficiency and the spectral response function mainly depends on the cathode type. The sensitivity variation between different tubes of the same type is usually in the same order as the differences between tubes of different types. The typical spectral response for the two cathode versions is shown right.

The dark count rate for the bialkali cathode is typically 20 to 80 counts per second. The mutialkali cathode usually has 200 to 600 dark counts per second.

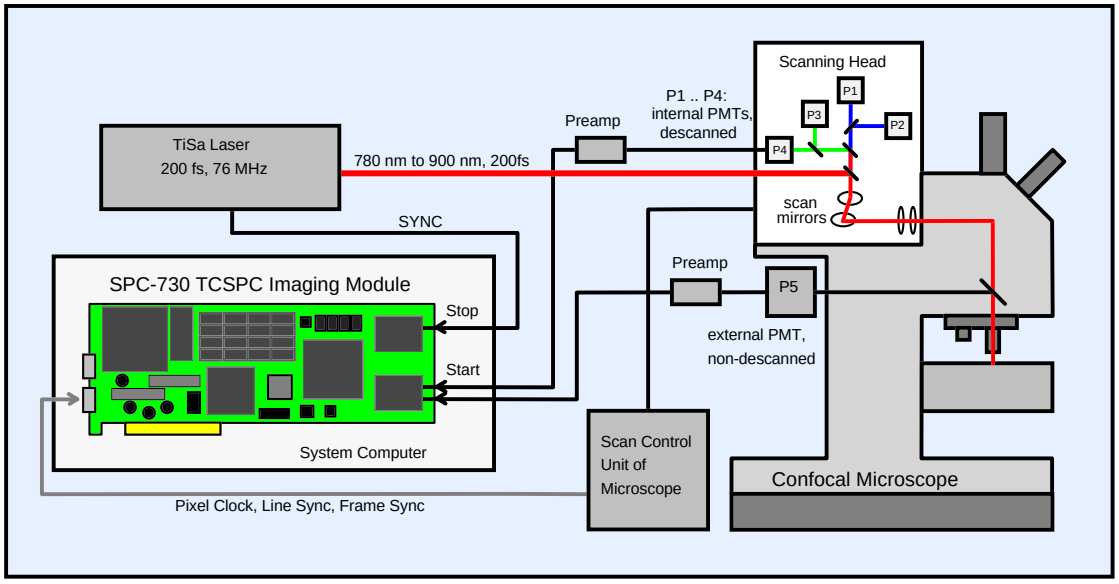
The sensitivity of the bialkali cathode drops sharply above 650nm. Therefore a 1 mm BG39 blocking filter is enough for this cathode. The multialkali tube has a sensitivity range up to 820nm and usually requires 3mm BG39. The figure right shows the spectral response of the bialkali cathode with 1mm BG39 and of the multialkali cathode with 3mm BG39.

Interestingly, there is almost no difference in the wavelenth range for the two cathode/filter combinations. It is probably better to sacrify a few nanometers in the red and take advantage of the higher blue sensitivity and the lower dark count rate of the bialkali cathode.



System Connections

The typical setup of the TCSPC microscope is shown in the figure below. A Ti:Sa laser delivers femtosecond pulses in the wavelength range from 780 nm to 950 nm.



General setup of the TCSPC Laser Scanning Microscope

The microscope scans the sample in the x-y plane providing an image in the focal plane of the objective lens. 3 D imaging is achieved by changing the depth of the focus in the sample.

Data acquisition is accomplished by the Becker & Hickl SPC-730 TCSPC Imaging module [1]. The CFD input receives the single photon pulses of the PMT. Synchronisation with the laser pulse sequence is achieved by the SYNC signal from the reference diode of the Ti:Sa laser.

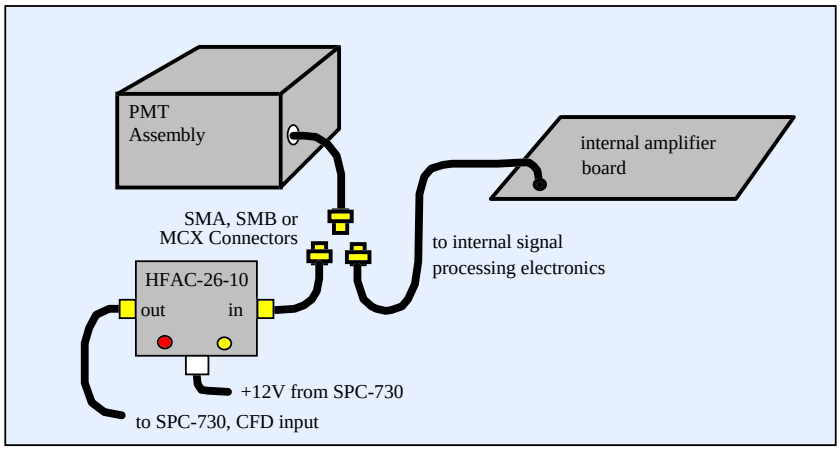
The SPC-730 module gets the scan control signals, Pixel Clock, Line Sync, and Frame Sync, from the scan controller of the microscope. Connecting the TCSPC module does not require any modification in the microscope electronics and software. The scan parameters are controlled in the usual way via the microscope software. The normal scan speed, the zoom function, region of interest setting, etc. can be used also when recording lifetime images. Furthermore, the SPC-730 can be run in parallel with the standard image recording electronics of the microscope. Basically, steady state images and lifetime images in different spectral ranges can be obtained at the same time.

Due to the simple interfacing the SPC-730 TCSPC imaging module can be adapted to almost any laser scanning microscope. The only requirement is that Frame Sync and Line Sync signals with TTL or CMOS levels can be made available. The Pixel Clock signal is not absolutely required. If a pixel clock is not available it can be generated in the SPC-730 module.

Detector Signals

Detectors in the Scanning Head

In most microscopes the internal PMTs are connected to preamplifiers designed for video signal bandwidth. The output signals of these amplifiers are too slow for photon counting. Therefore, the PMT output must be made directly available. The PMT output is always connected to a 50 Ω coaxial cable. Best case, there is a small coaxial connector (SMB, SMA, MCX, Lemo, etc.) that you can use to disconnect the PMT from the internal amplifier and to connect it via a HFAC-26-10 preamplifier to the SPC-730 module. Worst case, you have to desolder the cable either from the PMT assembly or from the internal amplifier board. We recommend to insert a connector into the cable so that you can easily connect the detector either to the scanning head electronics or to the SPC-730 system.



Warning: Do not connect or disconnect the PMT signal line when the PMT operating voltage is switched on. There is not only danger of electrical shock, you can also damage the preamplifiers.

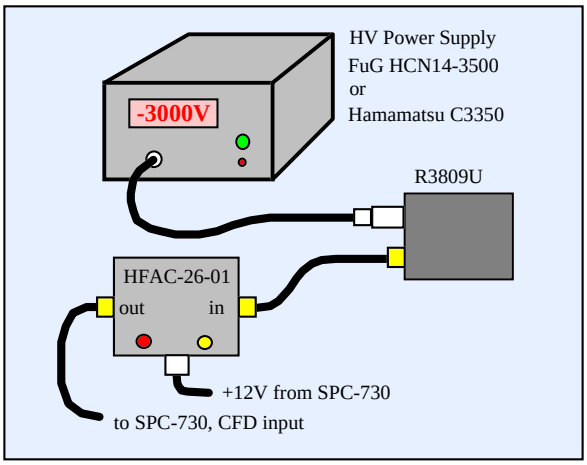
PMH-100 detector head

Connecting the PMH-100 is very simple. Connect its +12V power supply to pin 10 of the lower sub-D connector of the SPC-720 module. Connect a 50 Ohm SMA cable from the detector output to the CFD input of the SPC-730. You need not make the cables yourself, they are delivered with the PMH-100 if it is ordered together with the SPC-730.

The PMH-100 gives an optical and acoustical overload warning if the maximum output current of the PMT is exceeded. Please shut down the light or disconnect the +12 V immediately if you see the LED turning on or hear the overload beep.

R3809U MCP

The Hamamatsu R3809U MCP requires a high voltage power supply and an HFA-26-01 preamplifier. The connections are shown in the figure below.



Although the R3809U can be operated up to 3400 V a supply voltage of 3000 V is sufficient to get a system response below 30 ps and excellent counting efficiency. Therefore, an operating voltage of 3000V should not exceeded in order to achieve a maximum lifetime of the detector. Suitable power supplies are the Hamamatsu C3350 (available for 220 V and 127 V) and the FuG HCN14-3500. If you connect another power supply, please make sure that the output voltage is negative.

MCP PMTs are very sensitive to overload. The maximum permitted average output current is only 100 nA. Exceeding this value does not damage the MCP immediately but reduces the lifetime of the device if the overload persists for a longer time. The Becker & Hickl HFAC-26-01 preamplifier measures the output current of the MCP and turns on an overload warning LED if a current of 100 nA is exceeded.

Warning: Do not connect or disconnect the output signal line when the MCP operating voltage is switched on. There is not only danger of electrical shock, you can also damage the preamplifier. Please make sure that the connection between the MCP and the amplifier is reliable. Moreover, make sure that the HV cable and the HV connectors be in a good condition. Any interruption in the ground return path of the HV supply can put the detector case, the signal cables, the preamplifier, etc. on high voltage. Therefore be careful please, since touching 3000 V can ruin you the whole day.

Scan Control Signals

The scan control signal synchronise the data acquisition in the SPC-730 module with the scanning action of the microscope. Three signals are required:

- Frame Clock indicates the start of the next frame
- Line Clock indicates the start of the next line
- Pixel Clock indicates the start of the next pixel

The SPC-730 needs TTL or CMOS compatible pulses. The duration and the polarity of the pulses are not essential. The signals are connected to the upper sub-D connector of the SPC-730 module. The pin assignment of this connector is shown below.

1	+5V (max. 100mA)	**9**	**Line Clock**
2	Routing Signal, /R 7 or ARMED[2]	10	+12V (max. 60mA)
3	Routing Signal, /R 8 or TRGD[2]	11	-12V (max. 60mA)
4	Routing Signal, /R 9 or MEASURE[2]	**12**	**Pixel Clock**
5	**Ground**	13	TRIGGER[3]
6	-5V (max. 100mA)	14	CNTE2 (CNTE=CNTE1&CNTE2)
7	Routing Signal, /R 10	**15**	**Ground**
8	**Frame Clock**		

Frame Clock, Line Clock and Pixel Clock pulses are used in all scanning microscopes. The question is only whether they are available externally at an unused connector.

Cables for the Zeiss LSM-510 Axiovert and LSM-510 Axioplan and the Leica SP2 are available from Becker & Hickl. For other microscopes please contact Becker & Hickl under info@becker-hickl.com or phone +49 / 30 787 56 32.

Some microscopes have only the Frame Clock and the Line Clock available. In this case you can work with a pixel clock signal that is internally generated in the SPC module. This works since both the scanning speed of the microscope and the frequency of the synthetic pixel clock are constant. However, some microscopes use a non-uniform pixel clock to compensate for nonlinearity in the line scan. In this case you may find some distortion in the SPC image if you use a synthetic pixel clock.

If you make your own cable, please make sure that you don't accidentally connect the +12V or -12V of the SPC-730 to a scan control output of your microscope. This would almost surely damage the microscope electronics.

Synchronisation with the Laser

The synchronisation signal from the laser is required to provide a stop signal for the time measurement of the individual photon detection events (please see also SPC manual, [1]).

Most Ti:Sa lasers deliver a monitor signal that can be used for synchronisation. The SPC-730 module needs negative pulses of 100 to 500 mV amplitude and < 2ns risetime. The signal should have a stable amplitude and be free of AC components from the laser power supply and transients from the scanning head. If the pulses from your laser are positive, please use an inverting transformer available from Becker & Hickl.

If there is no suitable signal from the laser please use the PHD-400-N photodiode module of Becker & Hickl. A reflection of the Ti:Sa laser beam at a glass surface focused to the diode chip is sufficient to generate pulses of -100 mV (please see also SPC manual, [1]).

First Light

Detecting the first Photons

If you have a minimum of experience with optical detectors it should be no problem for you to put the SPC system into operation. In this case proceed as described below. Otherwise please suppress your aversion against manuals and read the section ‘Getting Started’ in the SPC manual [1].

Adjusting the Sync Signal

Start the SPC software. Set ‘Sync Threshold’ = -50mV, ‘Sync Zero Cross’ = -20mV, ‘Sync Frequency Divider’ = 4

If you use a photodiode module for synchronisation: Adjust the photodiode until 'SYNC OK' is displayed and the Sync rate corresponds to the repetition rate of your laser. If necessary, change ‘Sync Threshold’ and ‘Sync Zero Cross’. The current indicator at the photodiode module should go to about 10% of full scale for a 70 to 80 MHz laser. Caution: the current indicator of the PHD-100 is active only when the output is connected to the SPC module.

If the Sync signal comes from the laser: If necessary, change ‘Sync Threshold’ and ‘Sync Zero Cross’ until the displayed Sync rate corresponds to the repetition rate of your laser. Make sure that the pulses from the laser are negative.

Adjusting the detector

Do not give any light to your sample. Darken the room in order not to overload the detector by daylight leaking into the optical path.

Set ‘CFD limit low’ = 50mV, ‘CFD Zero Cross’ = 0

PMH-100 detector: Connect the +12V from the SPC card to the detector. Make sure that there is no overload warning (LED turned on or overload alert beep). You should see a CFD count rate of 100 to 500 counts per second. If you have a much higher count rate you probably have room light leaking into the detector.

R3809U MCP: Carefully increase the operating voltage. Watch the overload LEDs at the HFAC-26-01 amplifier. (Please make sure that you have the right amplifier. It must be the HFAC-26-**01** with 100nA overload current.) Stop the procedure if the LED turns on and find the way the daylight leaks into the detector. If everything is correct you should have a CFD rate of 100 to 500 counts per second at -3000 V.

Internal PMT: Proceed as described for the R3809U. The maximum supply voltage for the internal detectors is usually -900 to -1000V. The internal PMTs of some microscopes are prone to noise pickup from the scanning system. The effect shows up as a high count rate present only when the scanning is active. The count rate does not depend on the detector operating voltage. If you have effects like this, all you can do is to increase the CFD threshold until the false counts disappear and to operate the detector at a gain as high as possible.

When the detector voltage has been set up, start the SPC measurement in the Oscilloscope Mode. Use the parameters shown below:

System Parameters:	System Parameters:	Display Parameters:	Trace Parameters:
Operation Mode: Oscilloscope	TAC Range: 50ns	Scale Y: Linear	Trace 1: Active, Curve 1, Page 1
Overflow: Stop	TAC Gain: 1	Max Count: 65535	
Trigger: None	TAC Offset: 6%	Baseline: 0	
Coll Time: 0.5s	TAC limit Low: 8%	Point Freq: 1	
Display Time: 100s	TAC Limit High: 92%	Style: Line	
CFD Limit L: 50mV	ADC Resolution: 1024 or 4096	2D Display Mode: Curve	
CFD ZC Level: 0	Count Increment: 100		
SYNC: ZC Level -20mV	Memory Offset: 0		
SYNC Threshold: -50mV	Dith Rng: 1/16		
SYNC Freq Divider: 4	Routing Channels X,Y: 1		
Scan Pixels X,Y: 1	Page: 1		

As long as there is no excitation at the sample you should see virtually nothing except perhaps for some single photons scattered over the time axis. If you have more that a few thousand photons per second you are detecting daylight.

Give light to the sample. Be careful with the intensity. Even if you use one of the internal detectors of the microscope you may be surprised of the sensitivity of the PMT in the photon counting mode. On the screen you should see three or four subsequent signal periods of your fluorescence signal. Adjust the light intensity to a count rate of 100,000 counts per second or less.

Change CFD Limit Low. The count rate decreases with increasing threshold, but the shape of the system response improves. Set Limit Low that you get about 80% of the maximum count rate.

Adjusting time scale and delay

Set ‘Sync Frequency Divider’ = 1. It can happen that you see only a part of the decay curve now. Change the length of the SYNC cable until you see the decay curve well inside the last (right) 10 ns of the display window. 1 ns corresponds to a cable length of 20 cm. Alternatively you can change the length of the cable from the preamplifier or the position of the photodiode module in the optical path. Caution: Don’t connect or disconnect the PMT when the high voltage is switched on.

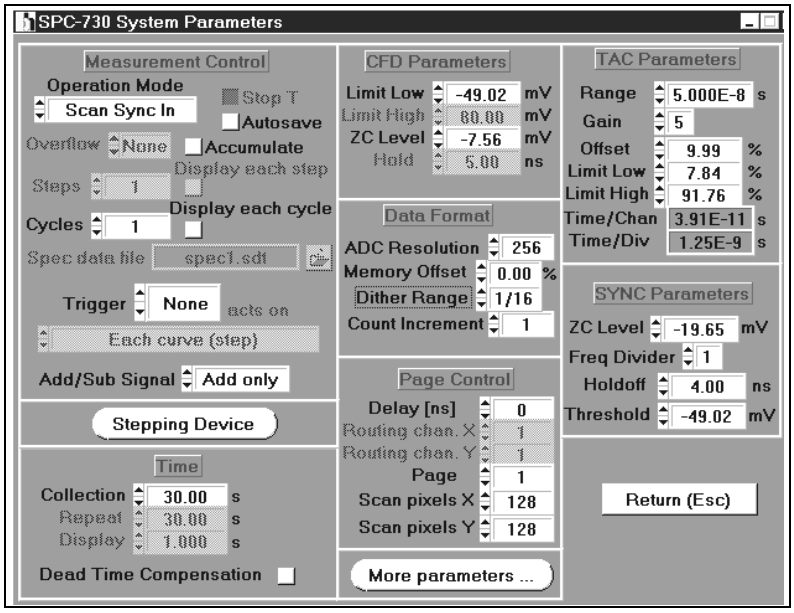
Increase ‘TAC Gain’ to stretch the decay curve over the full display window. The most appropriate gain is 5. This gives a display window of 10 ns and covers nearly one laser period.

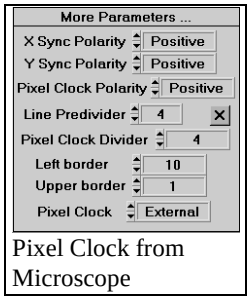
You can use a higher TAC gain to get finer time bins. Use ‘TAC Offset’ to centre the decay curve in the display window.

Save the result. Use ‘Main’, ‘Save’, options ‘SPC Data’, ‘All used data sets’. Advice: Please don’t name the file ‘test1.sdt’. Nine of ten files we receive for inspection are named ‘test1.sdt’ - you might get a strange answer.

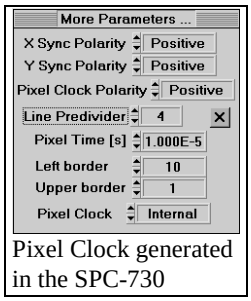
Recording the first image

If the scan control signals are not connected yet, connect them now. Except for the SYNC, CFD and TAC settings adjusted as shown in the last section, set the SPC-730 parameters as shown below:

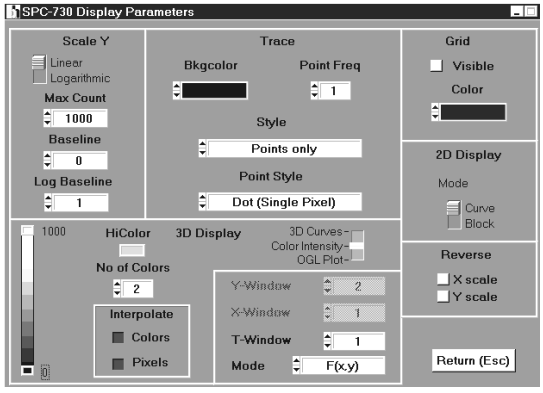


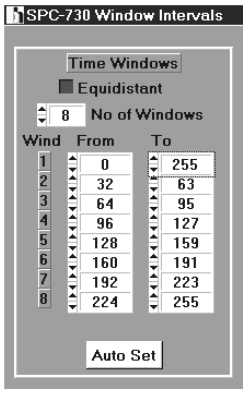


Pixel Clock from Microscope



Pixel Clock generated in the SPC-730





These settings give a full size TCSPC image of 128×128 pixels for a 512×512 resolution of the microscope. However, if you do not have a pixel clock from the microscope and thus have to generate the pixel clock in the SPC-730, the pixel time of 10 µs is only a rough estimate. It can happen that the real pixel dwell time is longer or shorter resulting in an image that is horizontally stretched or shrunk.

When all parameters are set, start the measurement and wait. The measurement should stop after the specified 'Collection Time', i.e. after 30 seconds. In practice it can take a few seconds longer because after the end of the collection time the acquisition is continued until the current frame is completed. If the measurement stops correctly, the frame clock, line clock and pixel clock signals arrive at the SCP-730 module. If the measurement does not stop one or several clock signals are missing.

If you do not see an image after the measurement has stopped, please reduce the 'Max Count' setting in the display parameters. Some image should become visible.

With the display parameter and window parameter configuration shown above, you get an image in the T Window 1 (specified in the 'Display Parameters'). This window covers all

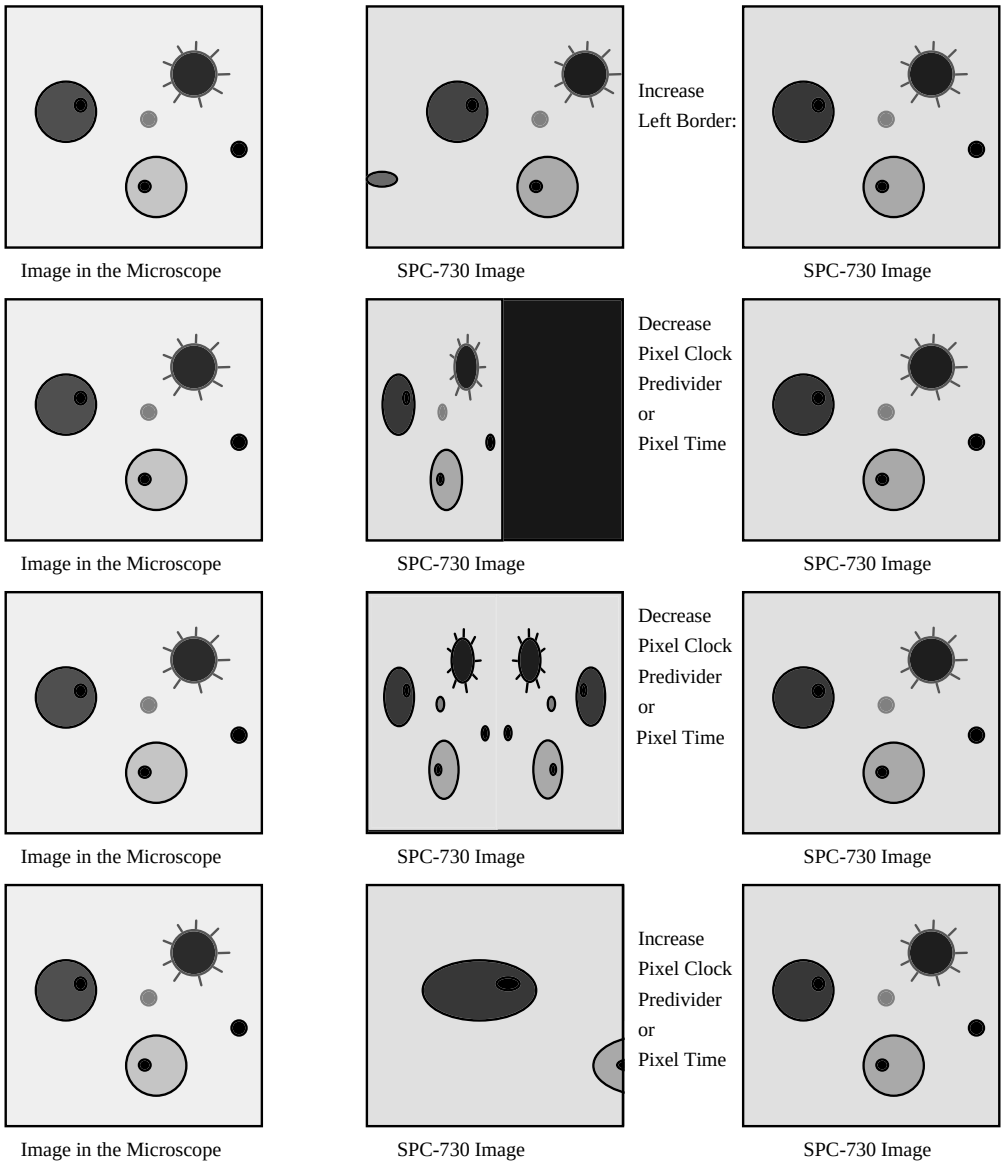
time channels of the decay curves stored in the individual pixels. Another seven T windows are defined in the Window Parameters. With the setting above, the T Windows 2 through 8 contain subsequent time intervals of the time axis. As you step through these T Windows by changing the T Window number in the display parameters you see the image appearing with the laser pulse and fading as the fluorescence decays.

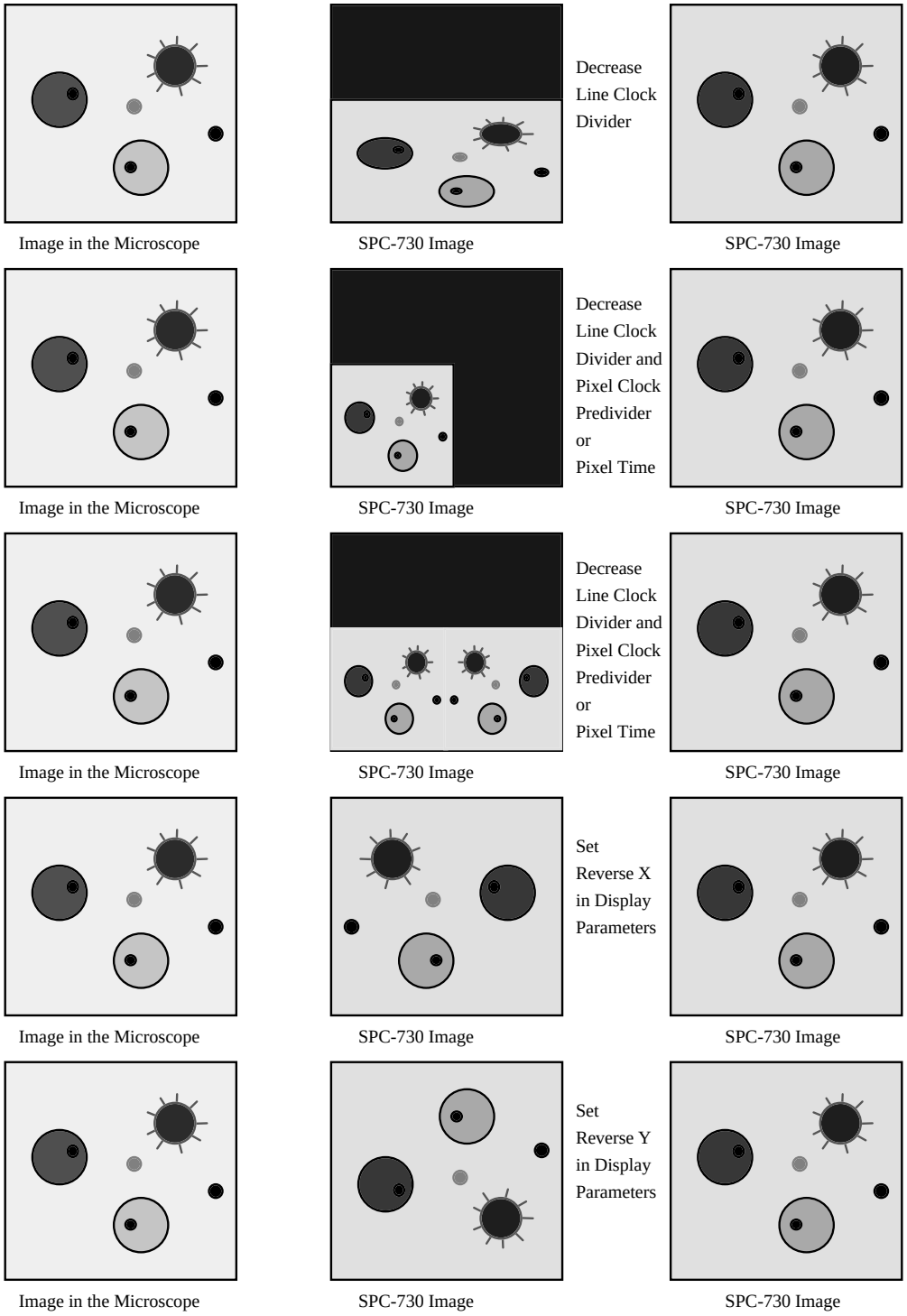
Do not forget to save the result. This saves also the setup parameters so that you can restore the system setup of the first successful measurement at any time.

If you do not get a reasonable image, please make sure that the parameters are set as shown above and check the scan control signals with an oscilloscope.

Adjusting image size and image location

The first images recorded with the settings shown under ‘Recording the first images’ can still have a wrong size, show a wrong part of the scan or be reversed in X or Y. Some typical effects and the way of correction are shown below.





Some microscopes, e.g. the older Leica SP1, deliver pixel clock pulses both at the start and at the end of each line. The result is an image as shown in the figure below. If you have effects like this please contact BH. We have adapters to transform odd scan control signals into useful frame and line pulses.

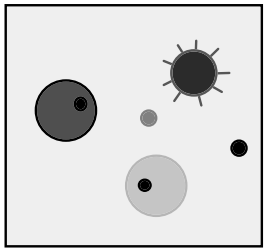
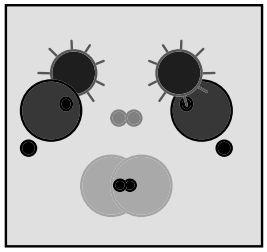
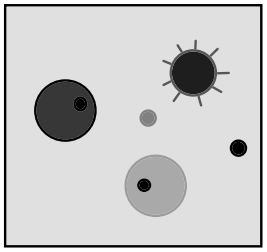


Image in the Microscope



SPC-730 Image

Contact
BH for
Adapter



SPC-730 Image

Assistance through bh

Software updates, new manual versions and application notes about new applications are available from our web site www.becker-hickl.de. Furthermore, we are pleased to support you in all problems concerning the measurement of fast electrical or optical signals. This includes discussions of new applications, the installation of the SPC modules, their application to your measurement problem, the technical environment and physical problems related to short time measurement techniques. Simply call us or send us an email.

Should there be a problem with your SPC module, please contact us. To fix the problem we ask you to send us a data file (.sdt) of the questionable measurement or (if a measurement is not possible) a setup file (.set) with your system settings. Furthermore, please add as much as possible of the following information:

Description of the Problem
SPC Module Type and Serial Number
Software Version
Type of the Microscope
Detector type, Operating voltage of the detector, PMT Cathode type
Preamplifier type, Gain, Bandwidth etc.
Laser System: Type, Repetition Rate, Wavelength, Power
SYNC Signal Generation: Photodiode, Amplitude, Rise Time
System Connections: Cable Lengths, Ground Connections. Add a drawing if necessary.
Environment: Possible Noise Sources
Your personal data: E-mail, Telephone Number, Postal Address

The fastest way is to send us an email with the data file(s) attached. We will check your system settings and – if necessary – reproduce your problem in our lab. We will send you an answer within one or two days.

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Literature

[1] SPC-134 through SPC-730 TCSPC Modules, Operating Manual. Becker & Hickl GmbH, www.becker-hickl.com.

[2] Photomultiplier Tube, Principle to Application. Hamamatsu Photonics 1994

[3] R3809U Series data sheet. Hamamatsu Photonics 1995, www.hamamatsu.com

[4] K. König, Multiphoton microscopy in life sciences, Journal of Microscopy 200 (1999) 1-23

[5] A. Schönle, Markus Glatz, Stefan Hell, Four-dimensional multiphoton microscopy with time-correlated single-photon counting, Appl. Opt. 39 (2000) 6306-6311

[6] W. Becker, A. Bergmann, K. Koenig, U. Tirlapur, Picosecond fluorescence lifetime microscopy by TCSPC imaging. Proc. SPIE 4262, 414-419

[7] K. König, P.T.C. So, W.W. Mantulin, B.J. Tromberg, E. Gratton, Two-Photon excited lifetime imaging of autofluorescence in cells during UVA and NIR photostress. J. Microsc. **183**, 197-204

[8] Femtolight series fiber lasers, IMRA America Inc., www.imra.com

[9] Pulsed diode lasers, PicoQuant GmbH, www.picoquant.com

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and Control Systems

Dec. 2001 Printer HP 4000 PS

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DCC-100
Detector Control Module

PCI compatible detector control module for single photon counting experiments

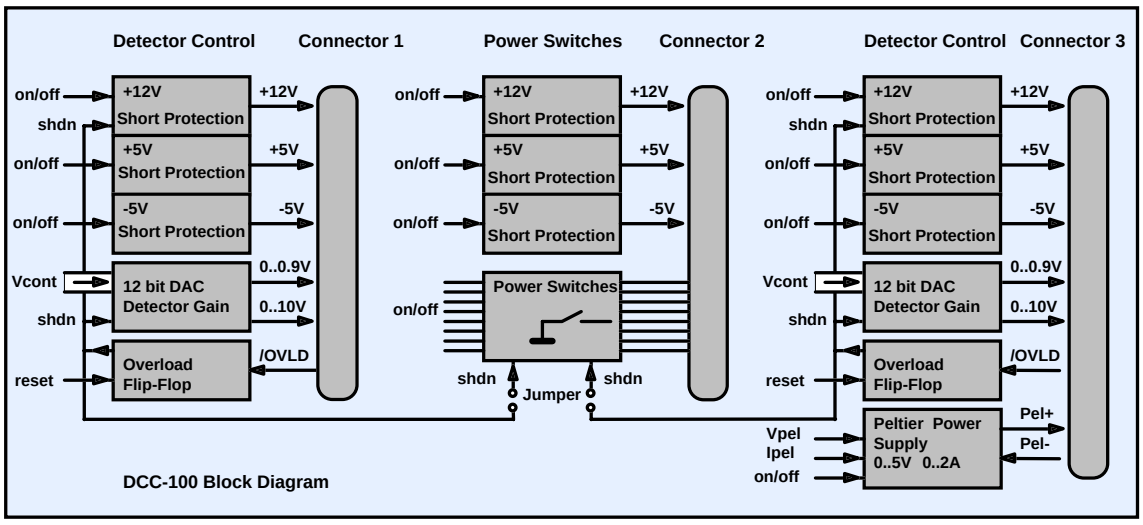
- Power supply and gain control for Hamamatsu H7422, H5783 or H5773modules
- Gain control for Hamamatsu R3809U MCP via FuG power supplies
- Overload shutdown of detectors in conjunction with bh HFAC-26 preamps
- Power supply for thermoelectric coolers, particularly for H7422
- Short circuit protected +12V, +5V and -5V power supply for preamps and detectors
- Software switched +12V, +5V and -5V power supply outputs for detector on/off control
- High current digital outputs for shutter and filter control
- PCI card, software for Windows 95, 98, 2000 and NT

Introduction

The DCC-100 module is used to control detectors in conjunction with **bh** photon counters. It can be used to control the gain of the Hamamatsu H7422, H5783, H6783 or similar photosensor modules by software. The gain of MCPs and PMTs can be controlled via the FuG HCN-14 High Voltage Power Supply. In conjunction with bh preamplifiers, overload shutdown of the detectors can be achieved. Furthermore, the DCC-100 delivers the current for thermoelectric coolers, i.e. for the Hamamatsu H7422. High current digital outputs are available for shutter or filter control. The DCC-100 is a PCI module for IBM compatible computers. It works under Windows 95, 98, 2000 and NT.

Structure of the DCC-100

The block diagram of the DCC-100 is shown in the figure below.



The DCC-100 contains two detector control blocks and one general purpose power switch block.

Detector control blocks

The detector control blocks contain power supply outputs for detectors, a digital-to analog converter (DAC) for detector gain control, and an overload shutdown circuit.

The power supply outputs are short-circuit-protected and deliver +12 V, +5 V and -5 V. The outputs can be switched on an off by software.

A 12 bit DAC is used to control the gain of the detector. It delivers the 0 to +0.9 V control voltage required for Hamamatsu photosensor modules (H7422, H5783, H57783 etc.) and a 0 to +10 V control voltage for FuG high voltage power supplies.

Both detector control block have a detector overload shutdown function. The shutdown function works in conjunction with **bh** HFAC-26 preamplifiers. If the preamplifier detects an overload condition it sends an active low overload signal (/ovld) to the DCC-100. This signal sets the overload flip-flop which shuts down the detector control voltage and the +12 V detector power supply. Furthermore, it can be used to deactivate the switches in the power switch block (see below).

One of the detector control blocks contains a power supply for thermoelectric coolers.

Power switch block

The power switch block contains power supply outputs for detectors and preamplifiers and eight high-current MOSFET switches to operate shutters, motors or magnetic actuators.

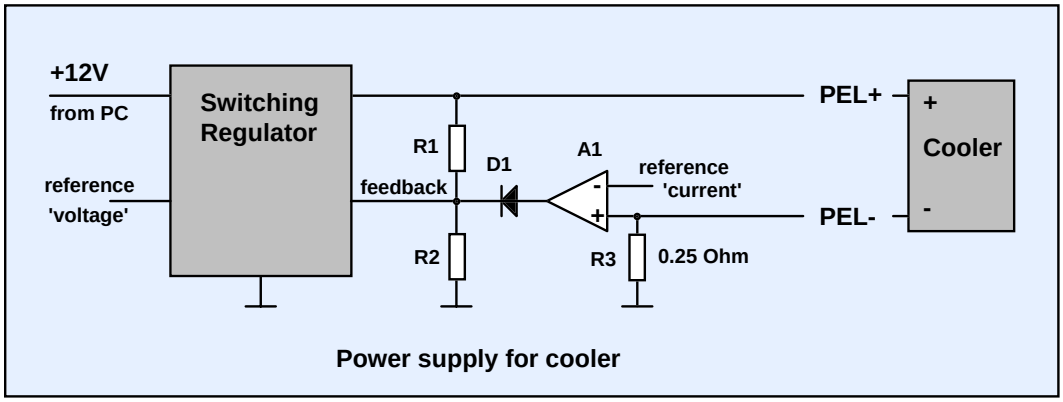
The power supply outputs are short-circuit-protected and deliver +12 V, +5 V and -5 V. The outputs can be switched on an off by software.

The MOSFET switches are able to switch currents up to 2A and voltages up to 20V. One side of each switch is connected to ground, the other side is available at the output connector. The switches are **not** short circuit protected.

The power switches can be shut down (i.e. set into the non-conducting state) by one or both overload signals from the detector control blocks. The configuration is set by jumpers on the DCC-100 board.

Power supply for thermoelectric cooler

Power supply for thermoelectric coolers is available at connector 3. The output can supply up to 5 V and 2 A. Both voltage and current can be selected by software. The device automatically controls either the output voltage or the output current. Voltage control is active as long as the current through the load is smaller than the current limit set by the software. When the current through the load reaches the current limit the device automatically switches to current control. A block diagram is shown in the figure below.



Power supply for cooler

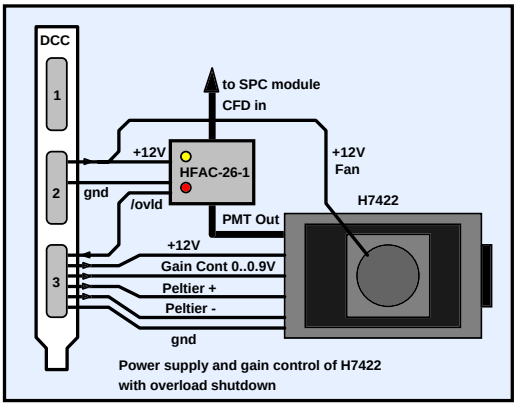
A switching regulator is used to generate the supply current for the cooler. The cooling current flows through the sensing resistor R3. As long as the voltage at R3 is smaller than the reference voltage at A1 (‘current’ reference) A1 is inactive and the switching regulator works as a normal voltage stabiliser. If the voltage at R3 exceeds the reference voltage at A1, the amplifier becomes active, and, via D1, overwrites the feedback voltage of the switching regulator. This causes the regulator to reduce the output voltage until the voltage at R3 equals the reference of A1.

Please note that the current control can work only if the cooling current flows back into the ’PEL-‘ pin of the DCC-100. Therefore make sure that the ‘-‘ side of the cooler is not connected to ground.

Applications

Some applications of the DCC-100 are described below.

Controlling the H7422



The H7422 is a high speed, high sensitivity PMT module of Hamamatsu. It contains a GaAs photomultiplier along with a thermoelectric cooler and a high voltage generator. The resolution in the TCSPC (time-correlated single photon counting) mode is typically 250 ps. The H7422 comes in different cathode versions for the wavelength range up to 1100 nm.

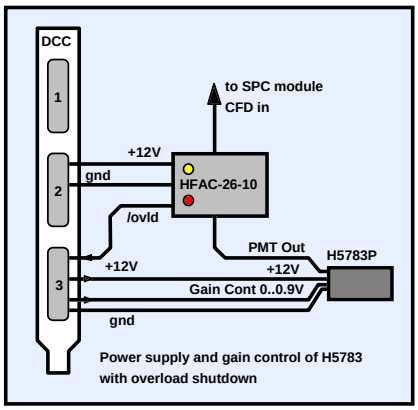
The recommended connection to the DCC-100 is shown in the figure right. The H7422 is connected to 'Connector 3' of the DCC-100. The DCC-100 delivers the +12 V operating voltage, the 0 to 0.9 V gain control voltage, and the current for the thermoelectric cooler. The output signal of the PMT is amplified by the HFAC-26-1 preamplifier. The single photon pulses at the output of the amplifier have a few 100 mV amplitude and are used to trigger the CFD input of a bh TCSPC module (SPC-300 through SPC-730, see manual at www.becker-hickl.de).

The HFAC-26-1 amplifier monitors the output current of the H7422. If an average current of 1 uA is exceeded the /ovld signal of the HFAC-26 goes to 'low'. This sets the overload flip-flop in the DCC, and the gain control voltage and the +12 V at connector 3 are shut down.

Notice: The H7422 has its own overload shutdown. The H7422 may shut down if the gain control voltage changes at a rate faster than 10 V/s. The changing rate of the gain control voltage in the DCC-100 is kept below the critical value. If the H7422 shuts down internally for whatever reason, it can be re-activated by cycling the +12 V operating voltage.

Don't connect or disconnect the signal cable from the detector to the preamplifier when the detector is switched on. This can destroy the amplifier. Make sure that the +12 V at connector 2 is switched on. Otherwise the HFAC does not work, and the overload protection is inactive.

Controlling H5783 and H5773 photosensor modules



The H5783 and the H5773 are high speed miniature PMT modules of Hamamatsu. The modules contain a small photomultiplier along with high voltage generator. The resolution in the TCSPC (time-correlated single photon counting) mode is typically 150 to 200 ps. The H5783 comes in various cathode versions for the wavelength range up to 820 nm. The H5783 can be controlled in a similar way as the H7422. The connections are shown in the figure right. A HFAC-26-10 preamplifier with a 10 µA overload threshold is used to amplify the single photon pulses and to send an overload signal to the DCC-100.

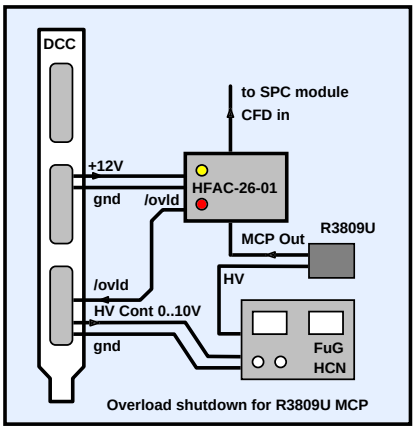
A second H5783 can be controlled separately via connector 1. In this case the power supply for both HFAC amplifiers should be derived from connector 2.

Don’t connect or disconnect the signal cable from the detector to the preamplifier when the detector is switched on. This can destroy the amplifier. Make sure that the +12 V at connector 2 is switched on. Otherwise the HFAC does not work, and the overload protection is inactive.

Controlling an R3809U MCP PMT

The Hamamatsu R3809U is the fastest photon counting detector currently available. It uses a microchannel plate for electron multiplication. In the TCSPC mode a time resolution of less than 30 ps (fwhm) can be achieved. MCP PMTs are extremely sensitive to overload. Because the microchannels are continuously destroyed by sputtering the lifetime of the detector is limited. The degradation effect is not noticeable under normal operating conditions. However, overloading the detector can rapidly exhaust the residual lifetime. Therefore, MCP PMTs should always be operated with overload protection, or at least with an overload indicator.

The figure right shows how an R3809U MCP can be operated with overload protection. An FuG HCN-14 is used as an high voltage power supply for the R3809U. The HCN-14 is available with a 0 to 10 V control input. The high voltage is proportional to the control voltage. Therefore, the detector operation voltage can be controlled by the DCC-100 and be shut down on overload.



For overload detection, the HFAC-26-01 preamplifier (with an overload threshold of 0.1 µA) is used.

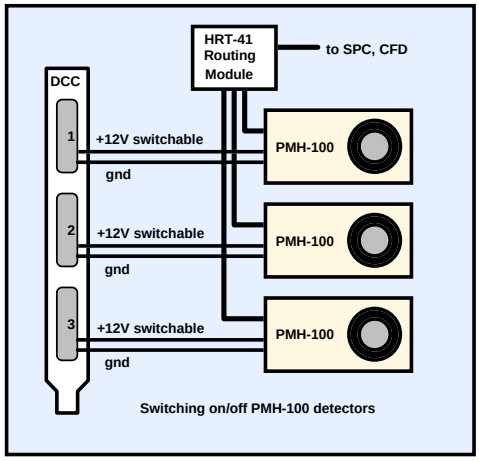
Notice: Please observe the usual safety rules when working with high voltage. Make sure that there is a reliable ground connection between the detector and the HV power supply. Don’t use broken cables, loose connectors or cables with insufficient insulation.

Don’t connect or disconnect the signal cable from the detector to the preamplifier when the detector is switched on. This can destroy the amplifier. Make sure that you connected all ground connections. Missing ground connections can result in an high voltage instability or unreliable overload shutdown.

Make sure that the +12 V at connector 2 is switched on. Otherwise the HFAC does not work, and the overload protection is inactive.

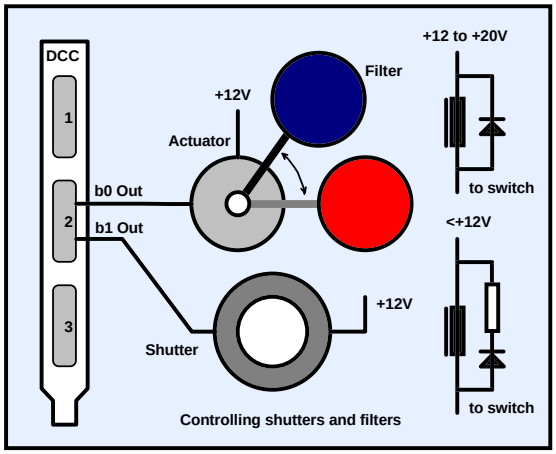
Controlling PMH-100 detectors

The PMH-100 of **bh** is a simple detector head that contains a Hamamatsu photosensor module, a preamplifier, and an optical and acoustical overload indicator. Since there is no overload signal available from the PMH-100 it cannot be automatically shut down on overload. Up to three detectors can, however, switched on and off via the DCC-100. By connecting the detectors to an **bh** SPC module via a HRT-41 or HRT-81 router, the signals of all detectors can be recorded simultaneously. Or, by switching only one detector on, the desired detector can be selected for measurement. The configuration is exceptionally useful for laser scanning microscopes which normally use several detectors in different light paths.



Controlling Shutters

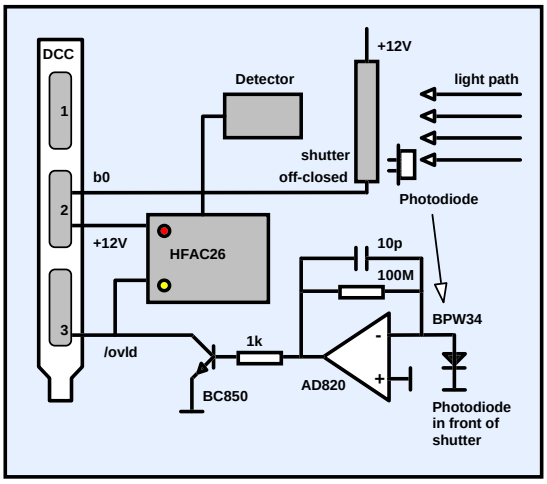
Shutters can be controlled via the power switches of connector 2. The switches can operate a voltage up to 20 V and up to 2 A. The power supply for the external load can be taken from connector 2 if a current of 100 mA at +12 V or 200 mA at +5 V is not exceeded. For higher currents an external power supply must be used.



Since shutters and other magnetic devices are inductive loads a flyback diode must be connected across the load. For supply voltage up to 12 V a resistor can be connected in series with the flyback diode to achieve a faster turn-off time. The value of the resistor should be about the DC resistance of the actuator or shutter coil.

The switches can automatically be shut down (i.e. set into the non-conducting state) when an overload condition occurs at connector 1 or connector 2. Which of the overload inputs is used for shutdown can be selected by jumpers on the DCC-100 board.

Sometimes it is required to lock a shutter in the ‘closed’ state as long as a potential overload condition persists. An example is a microscope with a mercury or halogen lamp. When the lamp is on, opening the shutter must be inhibited. A solution is shown in the figure right. The photodiode detects the light from the lamp, and the amplifier sends an ‘/ovld’ to the DCC-100. The signal can be connected parallel to the /ovld from the amplifier.



As long as the lamp is on, /ovld remains ‘low’ and the shutter cannot be opened. This gives additional safety against detector damage.

It must, however, be pointed out that an absolute safety against detector damage cannot be achieved in this way. If the lamp is switched on when the shutter is open it takes a few milliseconds until the overload is detected and the shutter closes. This can be enough to cause severe detector damage. Therefore, the best way is always an mechanical interlock so that the lamp path is closed when the detection path is opened.

Pin Assignment

Connector 1
15pin HD-SubD

Pin	Signal
1	+5V out switchable
2	
3	
4	
5	GND
6	-5V out switchable
7	
8	
9	
10	+12V out switchable, ovld sdwn
11	-12V out
12	0...+10V HV cont., ovld sdwn
13	0...+0.9V gain cont., ovld sdwn
14	/OVLD1 input
15	GND

Connector 2
15pin HD-SubD

Pin	Signal
1	+5V out switchable
2	Bit0 open drain out
3	Bit1 open drain out
4	Bit2 open drain out
5	GND
6	-5V out switchable
7	Bit3 open drain out
8	Bit4 open drain out
9	Bit5 open drain out
10	+12V out switchable
11	-12V out
12	Bit6 open drain out
13	Bit7 open drain out
14	
15	GND

Connector 3
15pin HD-SubD

Pin	Signal
1	+5V out switchable
2	Peltier +
3	Peltier +
4	Peltier +
5	GND
6	-5V out switchable
7	Peltier -
8	Peltier -
9	Peltier -
10	+12V out, switchable, ovld sdwn
11	-12V out
12	0...+10V HV cont., ovld sdwn
13	0...+0.9V gain cont., ovld sdwn
14	/OVLD3 input
15	GND

Installation

Requirements to the Computer

The computer must be a Pentium PC with a graphics card of 1024 by 628 resolution or more. There must be enough free PCI slots to insert the required number of DCC-100 modules. Although computer speed is usually not an issue for the DCC-100 we recommend a computer with a speed of at least 300 MHz.

Installation of the DCC-100 Software

The installation of the DCC-100 Standard Software is simple - start setup.exe from the installation disk.

You can install the software also from the Becker & Hickl web site, e.g. if you want to upgrade your system with a new computer and a new DCC software version has been released in the meantime. In this case proceed as described under ‘Update from the Web’.

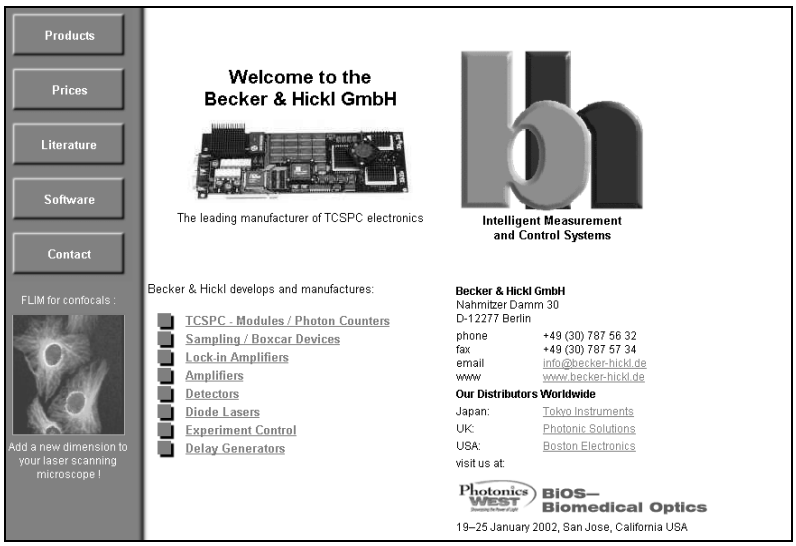
The DCC software is based on 'LabWindows/CVI' of National Instruments. Therefore the so-called 'CVI Run-Time Engine' is required to run the DCC software. The 'Run-Time Engine' contains the library functions of LabWindows CVI and is loaded together with the DCC software. The installation routine suggests a special directory to install the Run-Time Engine. If the required version of the Run-Time Engine is already installed for another application, it is detected by the installation program and shared with the existing LabWindows CVI applications.

Software Update

If you install a new DCC software version over an older one only the files are copied which have a newer date. This, to a certain extend, avoids overwriting setup files such as auto.set (the last system settings). Consequently, you cannot install an older software version in place of a newer one. If you want to do this (normally there is no reason why you should), run the ‘Uninstall’ program before installing.

Update from the Web

The latest software versions are available from the Becker & Hickl web site. Open www.becker-hickl.de, and click on ‘Software’.



Click on ‘Download’, ‘Windows 95/98/NT/2000’. Choose the DCC-100 software and get a ZIP file containing the complete installation. Unpack this file into a directory of your choice and start setup.exe. The installation will run as usual.

For a new software version we recommend also to download the corresponding manual. Click on ‘Literature’, ‘Manuals’ and download the PDF file. Please see also under ‘Applications’ to find notes about typical applications of the DCC in conjunction with **bh** photon counters.

Installation of the DCC-100 Module

To install the DCC-100, switch off the computer and insert the DCC module(s). To avoid damage due to electrostatic discharge we recommend to touch the module at the metallic back shield. Then touch a metallic part of the computer with the other hand. Then insert the module into a free slot of the computer.

The DCC-100 has a PCI interface. Windows has a list of PCI devices, and on the start of the system it automatically assigns the required hardware resources to the components of this list. When the computer is started first time with the DCC inserted Windows detects the DCC and updates the list of hardware components. Therefore it asks for driver information from a disk. Although this information is not actually required for the DCC you should select the driver information file from the driver disk delivered with the module.

If you don’t have the driver disk, please download the driver file from www.becker-hickl.com or www.becker-hickl.de, ‘Software’, ‘Windows 95/98/NT/2000’ or ‘Windows 3.1’, ‘Device drivers for **bh** modules’.

Boston Electronics Corporation, 91 Boylston St, Brookline MA 02445 USA * (800)3477-5445 or (617)566-3821 * fax (617731-0935 * tcspc@boselec.com

	SPC-630	SPC-730	SPC-830	SPC-134	Time Harp 200
Target Application	Standard lifetime experiments Single Molecule Detection Stopped Flow Correlation Experiments FCS Experiments	Standard lifetime experiments, Lifetime imaging, Confocal and two-photon scanning Microscopy Multi parameter experiments Stopped Flow	Standard lifetime experiments, Lifetime imaging, Confocal and two-photon scanning Microscopy Multi parameter experiments Stopped Flow Single Molecule Detection Correlation Experiments FCS Experiments	Optical tomography Single Molecule Stopped Flow Correlation Experiments FCS Experiments	Standard lifetime Single Molecule Microscope with scan stage Correlation Experiments FCS Experiments
No. of TCSPC Channels	1	1	1	4	1
Modules operable in parallel	4 x SPC-630	4 x SPC-730	4 x SPC-830	1 x SPC-134	
Conversion Principle	TAC - ADC with error reduction Patent DE 43 39 784 A1	TAC - ADC with error reduction Patent DE 43 39 784 A1	TAC - ADC with error reduction Patent DE 43 39 784 A1	TAC - ADC with error reduction Patent DE 43 39 784 A1	Time-to-Digital Converter
Detector Channel Sync Channel	Constant Fraction Constant Fraction	Constant Fraction Constant Fraction	Constant Fraction Constant Fraction	Constant Fraction Constant Fraction	Constant Fraction Level Trigger
Time Resolution	820 fs per time channel	820 fs per time channel	820 fs per time channel	820 fs per time channel	40 ps per time channel
Diff. nonlinearity	0.6% to 1% pp, <0.5% rms	0.6% to 1% pp, <0.5% rms	0.6% to 1% pp, <0.5% rms	0.6% to 1% pp, <0.5% rms	<6%pp, <0.5% rms
Detectable Lifetimes	2 ps to 2µs	2 ps to 2µs	2 ps to 2µs	2 ps to 2µs	<100ps to 4.5µs
Histogramming Process	Hardware, on board histogram memory	Hardware, 4-dimensional, on board histogram memory	Hardware, 4-dimensional, on board histogram memory	Hardware, on board histogram memory	Hardware, on board histogram memory
Image size for fast scan modes		max. 256 x 256 pixels	max 4096 x 4096 pixels		
Dead Time	125 ns	180 ns	125 ns	125 ns	<350 ns
Useful continuous count rate, Histogram Modes, 50% loss, per module	4 MHz	2.8 MHz	4 MHz	16 MHz (overall for 4 channels)	1.4 MHz
Peak Count Rate, histogram modes, 50% loss, per moduel	4 MHZ	2.8 MHz	4 MHz	16 MHz (overall for 4 channels)	1.4 MHz
Continuous count rate, time-tag modes	0.4...0.8 MHz, depends on computer speed and background activity		3...4 MHz, depends on computer speed and background activity	0.4...0.8 MHz, depends on computer speed and background activity	Depends on computer speed and background activity
Peak count rate, time-tag modes, 50% loss	4 MHz independent of computer speed		4 MHz independent of computer speed	16 MHz independent of computer speed	Depends on computer speed and background activity
on-board FIFO buffer size, time tag modes	128,000 photons or 256,000 photons		8 Million photons	512,000 photons	128,000 photons
Macro time resolution in time tag (FIFO) modes	50 ns		50 ns from internal clock or 12ns to 100 ns from sync (laser)	50 ns from internal clock or 12ns to 100 ns from sync (laser)	100ns
Scan rate, Scan syn in mode		down to 100ns per pixel independent of computer speed	down to 100ns per pixel independent of computer speed		
Multi-Detector Operation	yes Patent DE 43 39 787 A1	yes Patent DE 43 39 787 A1	yes Patent DE 43 39 787 A1	yes Patent DE 43 39 787 A1	yes
No of curves in memory	2 x 64 to 4096	1024 to 65,536	4096 to 2,000,0000	2 x 32 to 2 x 2048 per TCSPC channel	2 x 32
Min. time per histogram	1µs in continuous flow mode	100ns in scan sync in/out mode	100ns in scan sync in/out mode	1µs in continuous flow mode	1µs in ext sync mode

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	SPC-630	**SPC-730**	**SPC-830**	**SPC-134**	**Time Harp 200**
Available multi-detector extension devices for	4 MCPs, 4 PMTs 8 MCPs, 8 PMTs 8 APDs 16 channel pmt head	4 MCPs, 4 PMTs 8 MCPs, 8 PMTs 8 APDs 16 channel pmt head	4 MCPs, 4 PMTs 8 MCPs, 8 PMTs 8 APDs 16 channel pmt head	4 MCPs, 4 PMTs 8 MCPs, 8 PMTs 8 APDs	4 APDs
Operating Modes	Single Oscilloscope 2 dimensional f(xyt) Sequence f(t,T), fi(t,ext) Spectrum fi(T), fi(ext) Continuous Flow (unlimited seq.) Time Tag (FIFO)	Single Oscilloscope 2 dimensional f(xyt) Sequence f(t,T), fi(t,ext) Spectrum fi(T), fi(ext) Imaging (Sync In, Sync Out, XY in, XY out)	Single Oscilloscope 2 dimensional f(xyt) Sequence f(t,T), fi(t,ext) Spectrum fi(T), fi(ext) Imaging (Sync In, Sync Out, XY in) Time Tag (FIFO)	Single Oscilloscope 2 dimensional f(xyt) Sequence f(t,T), fi(t,ext) Spectrum fi(T), fi(ext) Continuous Flow (unlimited seq.) Time Tag (FIFO)	Integration Oscilloscope Sequence f(t,T) Contiuous Time-tag (Option)
Experiment Trigger	Start of measurement Start of sequence Each step of sequence	Start of measurement Start of sequence Each step of sequence Frame Clock, Line Clock, Pxl Clock	Start of measurement Start of sequence Each step of sequence Frame Clock, Line Clock, Pxl Clock	Start of measurement Start of sequence Each step of sequence	Start of measurement Start of sequence
Triggered accumulation of sequences	yes	yes		yes	yes
Detector / Experiment control (Own products only)	Preamplifiers with detector overload protection, PMH-100 Detector modules, PML-16 multichannel detector head, DCC-100 Detector Controller , STP-340 Step Motor Controller, Routers for MCPs, PMTs, APDs, Dual ADC module for XY In operation	Preamplifiers with detector overload protection, PMH-100 Detector modules, PML-16 multichannel detector head, DCC-100 Detector Controller , STP-340 Step Motor Controller, Routers for MCPs, PMTs, APDs, Dual ADC module for XY In operation, Adapters for Zeiss, Leica, Olympus and Biorad laser scanning microscopes	Preamplifiers with detector overload protection, PMH-100 Detector modules, PML-16 multichannel detector head, DCC-100 Detector Controller , STP-340 Step Motor Controller, Routers for MCPs, PMTs, APDs, Dual ADC module for XY In operation, Adapters for Zeiss, Leica, Olympus and Biorad laser scanning microscopes	Preamplifiers with detector overload protection, PMH-100 Detector modules, PML-16 multichannel detector head, DCC-100 Detector Controller , STP-340 Step Motor Controller, Routers for MCPs, PMTs, APDs	Preamplifiers with detector overload protection, Routers for APDs
Free Documentaion available on web site	SPC Manual, 165 pages; TCSPC Introduction, 5 pages; Upgrading laser scanning microscopes for lifetime imaging; Controlling SPC modules; Protecting Photomultipliers; FRET measurements by TCSPC lifetime microscopy; Multi-wavelengthe TCSPC lifetime imaging; High count rate multichannel TCSPC for optical tomography; Optical Tomography: TCSPC Imaging of Female Breast; Setting up High Gain Detector Electronics for TCSPC Applications; Testing SPC Modules; 16 Channel Detector Head for TCSPC Modules; Routing Modules for Time-Correlated Single Photon Counting; Detector Control Module DCC100 Manual; **TCSPC Software is available and FREE**; Manual: Multi - SPC 32 bit Dynamic Link Library				Measurement examples
Related Products (Own products only)	SPC-300, SPC-330 TCSPC; SPC-400, SPC-430 TCSPC; SPC-500, SPC-530 TCSPC; MSA-100 1ns multiscaler; MSA-300 5ns multisclaer; PMS-400 and PMM-328 Gated photon counters / multiscalers; Picosecond Diode Lasers				Time Harp 100 Picosecond Diode Lasers