

TILL Photonics

# Photometry Manual

## 1.1 CONTENTS

1.1	CONTENTS.....	2
1.2	OVERVIEW .....	3
1.3	Survey of Combinations of T.I.L.L. Photometry Equipment .....	4
	.....	4
1.4	Installation.....	5
1.5	ADC-board.....	5
1.5.1	Getting started with the ADC.....	6
1.5.2	Functions of the ADC .....	6
1.5.3	Communication via RS-232 using the ADC Communication Software .....	7
1.5.4	Complex Functions of the ADC.....	11
1.6	.....	13
1.6	FDU: Fluorescence Detection unit, dual excitation / single emission.....	13
	Signal flow in the fluorescence detection unit equipped with integration.....	14
1.7	.....	15
1.7	Photodiode detector head .....	15
1.8	Viewfinder III: .....	16
1.8.1	Viewfinder Features.....	16
1.8.2	Schematic of ViewFinder III:.....	17
1.8.3	Installation.....	17
1.8.4	Filter K65:.....	17
1.9	Photomultiplier detector head .....	18
1.9.1	Installation.....	18
1.10	Using your photometry setup with HEKAs Fura extension.....	18

## 1.2 OVERVIEW

The T.I.L.L. Photonics **Photometry System** allows to follow changes in biological activity on a **millisecond time scale** assayed by a variety of fluorescence indicators.

The integrated system is available as **Dual Excitation** (up to two excitation wavelengths) / **Single Emission** (one emission wavelength) or as **Single Excitation / Dual Emission** System (Other special configurations can be done upon request). The fluorescence signals can be sampled **unprocessed** or as already **integrated** signal from 2 analogue output channels. This way the system is easily interfaced to programs for electrophysiology like the HEKA Pulse or X-Chart software, P-Clamp by Axon Instruments or your own software.

In the most highly integrated version of the Photometry System you can control a pre-defined wavelength protocol with a TTL level or via RS-232 interface. Due to the fact that the **POLYCHROME II** can reach every wavelength in a wide range, with a little effort it is possible to do **multi excitation** experiments. If you use for example your own DA-converter to control the wavelength of the **POLYCHROME II** it is possible to excite various different wavelengths and detect the raw fluorescence data simultaneously.

In the following you will find a description of our photometry equipment which is normally a part of the **POLYCHROME II** illumination system (Please refer to the corresponding manual). The “names” ADC, FDU etc. denote plug-in boards in the control unit of the Polychrome II.

*Please note that in most cases your customised equipment may contain only some of the parts mentioned.*

The abilities of the most suitable combinations are listed in short in the following table. A detailed description on “how to use these combinations” is given in the following chapters.

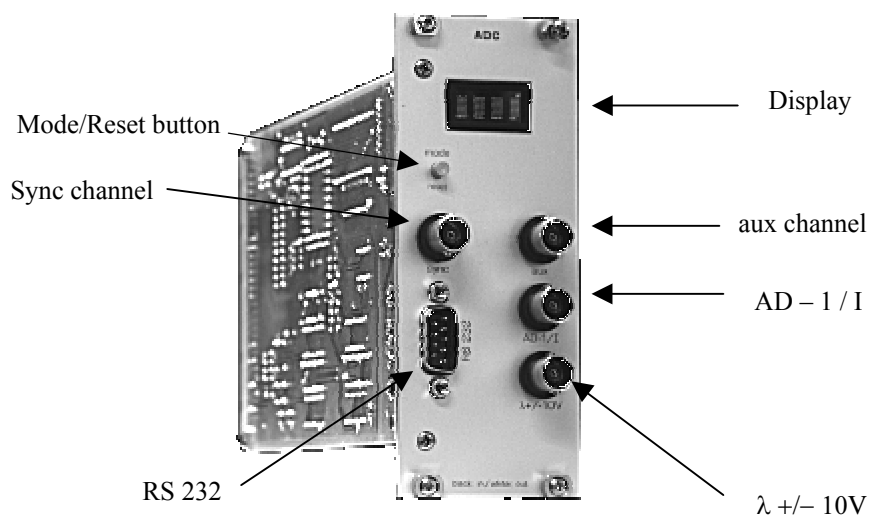
### 1.3 Survey of Combinations of T.I.L.L. Photometry Equipment

Application	Minimal Hardware-requirements	Comment
<b>Photometry in combination with electrophysiology</b>		
<b>- single emission systems</b>		
Single/dual excitation, single emission, raw signal output	ADC, FDU, photodiode or photomultiplier, optional viewfinder	To use with HEKA's <b>fura extension</b> , etc.
Single/dual excitation, single emission, raw or integrated signal output	ADC, FDU with integrator, photodiode, optional viewfinder	To use with Axon's P-Clamp, HEKA's X-Chart, etc.
<b>- dual emission systems</b>		
Single/dual excitation, dual emission, raw or filtered signal output	ADC, FDU, photodiodes, optional viewfinder	
Single excitation, dual emission, raw or filtered and integrated signal output	ADC, FDU with integrator, photodiodes, optional viewfinder	To use with Axon's P-Clamp, HEKA's X-Chart, etc.
<b>Photometry in combination with imaging</b>		
<b>- single or dual emission</b>		
Raw or filtered signal output	FDU, photodiode(s)	To use with HEKA's <b>fura extension</b> , etc.. Utilises the ISC of the imaging system for wavelength control.
Integrated and optionally filtered output	FDU with integrator, ADC, Control Unit, photodiode(s)	To use with Axon's P-Clamp, HEKA's X-Chart, etc..
<b>Photometry "stand alone"</b>		
Single or dual emission measurements, up to two excitation wavelengths. Integrated signal output.	ADC, FDU with integrator, photodiode(s), optional viewfinder, ITC-16 interface board, X-Chart software.	Other external hard- and software for data acquisition possible
<b>Photometry "stand alone" without polychrome II</b>		
Single/dual emission measurements. Raw signal output.	Control unit, FDU, photodiode(s), optional viewfinder	Hard- and software for data acquisition must be available. Software must be able to separate channels in dual excitation mode.

## 1.4 Installation

- If you are upgrading your system, remove the ASC board from the Control Unit and plug in the ADC board to the free slot.
- For **external** wavelength control by an analogue voltage, connect the BNC carrying the  $\lambda$  control voltage ( $\pm 10$  V) with the BNC  $\lambda \pm 10V$  on the front panel of the ADC.
- For communication via RS-232, connect the serial port of the PC with the 9-pin plug-in (**RS-232**) on the front panel of the ADC. For control of the wavelength protocol, connect a TTL line to the **sync** channel on the front panel of the ADC.
- Connect the photodiode to the **detector** plug on the front panel of the FDU.
- Connect the left **out** plug to the analog input of your data acquisition system.

## 1.5 ADC-board



### Purpose:

The ADC board sets the wavelengths of the monochromator and controls the execution of simple protocols sent by a computer via the RS 232 interface. This is achieved with the help of a micro-controller which is supported by some programmable devices. With this solution control of the integration of the fluorescence signal is independent of the time scale of the computer.

### Description of the plugs:

**Sync channel:** to be connected via a BNC cable to a triggering port. Via this port the execution of a pre set wavelengths protocol can be triggered.

**RS 232:** to be connected via a zero modem cable to a RS 232 port of the computer: Baud Rate: 19200, Data Bits: 8, Parity: None, Stop Bits: 1, Flow: XON/XOFF. The RS 232 port is used to control the monochromator and to transfer protocols from the imaging computer to the ADC board.

**AD 1 / I:** During the calibration of the monochromator the photodiode has to be connected to this port. The internally connected current/voltage converter with the following analog digital converter converts the photo-current of the photodiode to a digital number. The intensity of the monochromator is measured with the photodiode through the supplied interference bandpass filters. During the calibration the ADC board scans the monochromator through the total wavelength range while taking the reading of the photodiode. At the maximum reading of the photo-current the internally supplied analog voltage corresponds to the wavelength of the interference filter.

**$\lambda$  +/- 10V:** The monochromator can be controlled with an external analog voltage within the range of +/- 10 Volts: If an analog voltage is supplied at that port the wavelength of the monochromator is controlled by that voltage. Minus 10Volts corresponds to the longest possible wavelength, plus 10 Volts to the shortest possible wavelength of the monochromator.

**Aux:** Connects to the ISC if imaging and photometry with integration are combined.

**Please be careful not to supply a voltage higher than plus 10Volts or lower than minus 10Volts.**

#### 1.5.1 Getting started with the ADC

After switching on the **DC Power** of the Polychrome II in the display of the ADC the lamp hours appear for about 15 seconds (e.g. h012 = 12 hours). Then, the ADC will switch to the mode activated before the previous shut down of the DC power. (A new ADC should stay in the lamp hour mode.)

Pressing the **mode/reset** button switches to external wavelength control (Display: extn). In this mode the  $\lambda$ +/- 10V channel is activated and the wavelength is controlled by the analogue voltage applied to the plug. To activate other programs press the **mode/reset** button again.

#### 1.5.2 Functions of the ADC

In the following table you will find a description of the different functions of the ADC.

Function	Display
<b>External wavelength control:</b> The $\lambda$ +/- 10V channel is activated for wavelength control.	Extn
<b>Photo Current:</b> Display of the current measured from the PhotoCurrent input channel AD1/I. One display unit corresponds to about 3 $\mu$ A current.	i000-i999
<b>CurrentSpike peak measurement:</b> Display of the peak amplitude of a current spike measured from the PhotoCurrent input channel AD1/I. Spike detection is activated if the signal crosses a threshold. The spike is terminated when the signal falls below the threshold. The peak value is displayed until the threshold is crossed again. One display unit corresponds to about 3 $\mu$ A current amplitude.	s000-s999

Function	Display
<b>Wavelength Protocol:</b> Run/Stop control of the default wavelength protocol via BNC connector (sync). Set sync to high level (+5V) to run wavelength protocol. Low (0V) will stop the wavelength protocol.	wait (TTL low signal on sync) wrun (TTL high on sync channel, protocol execution)
<b>Definition of the wavelength protocol:</b>	
<p> wl 0: resting wavelength  wl 1, wl 2: two excitation wavelength (default: 340 nm / 380 nm)  dt : dead time (default 2.5 ms)  it 1, it 2 : integration time for wl 1 and wl 2 (default 7.5 ms)  rt: resting time  <math>\Delta t</math> : cycle time, sum of: <math>dt+it_1+dt+it_2+rt</math> </p> <p>All times are in units of 100<math>\mu</math>s (valid values 2 .. 56000) and wavelength in nm.  Trigger 1 and trigger 2 will synchronise the integrator on the FDU (fluorescence detection unit).</p>	

### 1.5.3 Communication via RS-232 using the ADC Communication Software

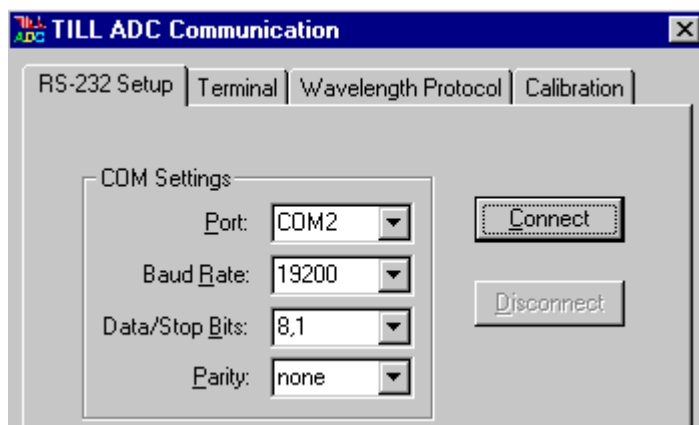
If you use a PC with a Win9x/NT operating system you can use our ADC communication software which allows a comfortable control of the ADC.

#### RS-232 Setup

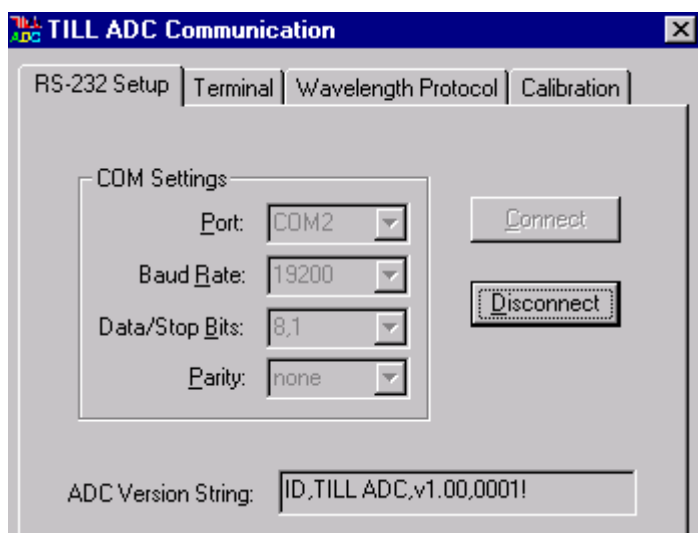
After the start of the ADC Communication program you have to setup a connection with the ADC:

1. Setup a hardware connection from a serial port of your PC to the RS-232 connector at the ADC front panel (zeromodem cable) and turn on the power of the Control Unit.
2. Specify the following COM settings in the dialog:

Port	Serial Port # (COM1-COM3)
Baud Rate	19200
Data/Stop bits	8,1
Parity	None

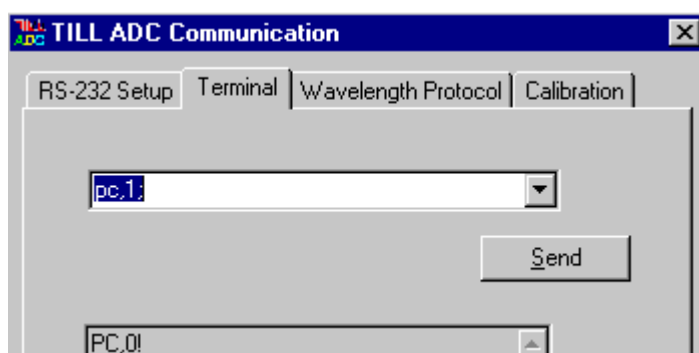


3. Press the **Connect** button. A successfully established connection will be indicated by the display of the ADC Version string. A connection failure occurred when the ADC Version string is **unavailable**.
4. The ADC Version String indicates the identification code of ADC hard- and software.



5. To disconnect the ADC Communication program press the **Disconnect** or **Close** button.

### Terminal



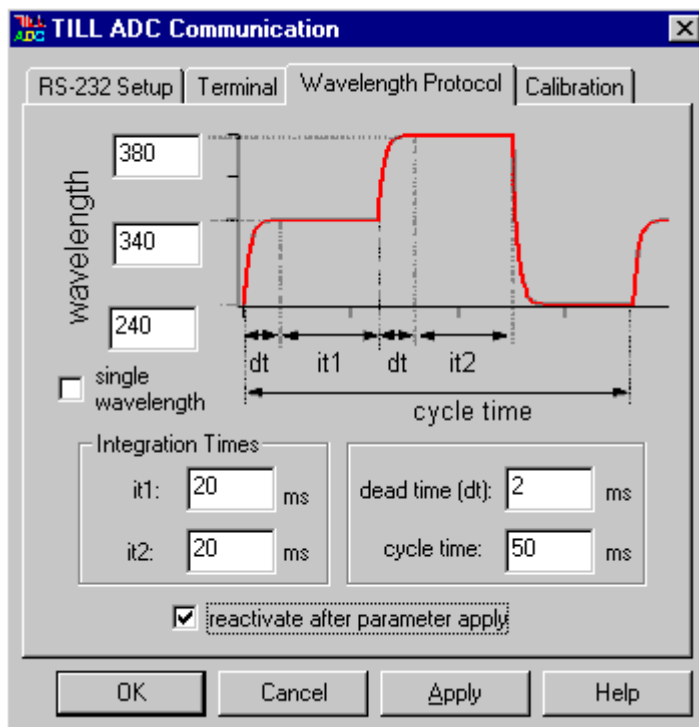
Enter a command string in the upper line or use the pull down menu to select a command sent previously. With the **Send** button the string will be send to the ADC. The answer string coming from the ADC will be displayed in the field below the entry line.

The following list contains the valid commands:

String sent to ADC	Answer from ADC	Comment
ID;	ID, name, version No., serial No.!	Identification of hard and software
SR,0;	SR,0!	Set Relais. 0 = control via RS-232, 1 = analog control by external voltage
WL,340; <i>or</i> WL,0;	WL,0!  WL,240!	Sets wavelength to 340 nm  To set and get the minimum wavelength.
WA,1,437;	WA,0!	Write Analog OUT port, Port, value in counts (pseudo 12 bit). Port #1 is the channel for wavelength control.
RA,1;	RA,1,257!	Read analog IN port, port, value in counts (pseudo 12 bit). Port #1 is the PhotoCurrent channel AD1/I.
RC;	RC,380,700,505,1700!	Read Calibration WL1, DAC1, WL2, DAC2
WC, 380, 700, 505, 1700;	WC,0!	Write Calibration WL1, DAC1, WL2, DAC2
PE,1;	PE,0;	Mode: Wavelength protocol
PE,2;	PE,0;	Mode: PhotoCurrent display
PE,3;	PE,0;	Mode: Spike display
DT, 345;	DT,0!	Define Threshold, threshold in counts
PD,20,200,250,2000,260,340,380;	PD,0! Ok PD,1! Definition error	Definition wavelength protocol: Dt, it1, it2, rt, w11, w12, w13 Omit it2 and w12 to define a single wavelength protocol
RP;	RP,2,20,25,201,200,340,380!	Read protocol parameter
PC,0;	PC,0!	Start wavelength protocol
PC,1;	PC,0!	Execute wavelength protocol when sync is high.
PR,50,0;	PR,0!	Execute wavelength protocol 50 times
PR,50,1;	PR,0!	Execute wavelength protocol 50 times when sync is high.
PS;	PH,0!	Stop wavelength protocol
RS;	RS,0!	reset processor

## Wavelength Protocol

When opening the Wavelength Protocol window the active protocol settings are displayed. You can change each parameter by editing the referring value.



When selecting the check box **single wavelength** the parameters  $it_2$  and  $wl_2$  are disabled - a single wavelength protocol will be applied. When using these options in combination with the hardware integration of the fluorescence signals on the TILL FDU board, both channels integrate in parallel.

Table of valid protocol parameter:

Dead time (dt)	0.2 - 5600 ms (suggested 2.5ms)
Integration time 1 ( $it_1$ )	0.2 - 5600 ms (suggested 10ms)
Integration time 2 ( $it_2$ )	0.2 - 5600 ms (suggested 10ms)
cycle time	0.2 to 5600ms+2*dt+ $it_1$ + $it_2$
Wavelength 1 ( $wl_1$ )	Minimal – maximal wavelength
Wavelength 2 ( $wl_2$ )	Minimal – maximal wavelength
Resting wavelength ( $rw_1$ )	Minimal – maximal wavelength (suggested appr. 260nm)

### Comments:

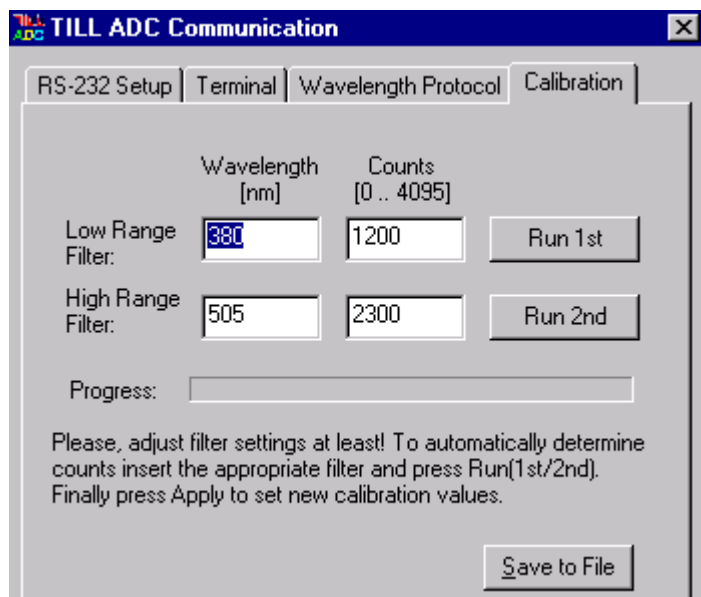
The valid times taken from the ADC are 0.2-5600ms. Since the cycle time is the sum from  $2*dt + it_1 + it_2 +$  resting time, the limiting value for slow repetition rates is the resting time.

Minimal and maximal wavelength are parameters which depend on the active calibration. To determine the minimal and maximal wavelength use the WL,0; to get the minimum (Answer e.g.: WL,259!) and WL,999; to get the maximum.

Select the check box **reactivate after parameters apply** when the ADC should continue with its last task after applying new protocol parameters.

## Monochromator Calibration

When opening the Calibration window the active calibration parameters are displayed.



**Manual recalibration:** Edit the wavelength/DAC count pairs and press **Apply**.

**Automatical recalibration:** Adjust filter settings for your calibration filters. Connect the photodiode of the calibration set to the AD-1/I channel on the ADC front panel. Insert low range filter (filter 1) and press **Run 1st**. Then, insert the high range filter (filter 2) and press **Run 2nd**. The peak DAC values will be displayed following the wavelength scan. Press **Apply** to set new calibration parameters.

The values should be in the range of **1000** and **1500** for the 380 nm filter and between **2000** and **2500** for the 505 nm filter.

You can use the option **Save to File** to save the latest scan to a text file.

### 1.5.4 Complex Functions of the ADC

**Note:** These functions can be performed more comfortable with the ADC communication program.

#### Recalibrate the monochromator with external wavelength control:

Connect the Photodiode of the Calibration Set with the AD-1/I channel of the ADC front panel.

1. Activate the program PhotoCurrent Display on the ADC by pressing the mode/reset button until the letter i and 3 digits appear in the display.
2. Insert filter #1 (380 nm) into the photodiode holder. Aim the light coming out of the light guide onto the photodiode.
3. Adjust the analog voltage on the  $\lambda$ -in channel until the photo current reaches its maximum. Write down the analog input voltage:  $V_1$
4. Insert filter #2 (505 nm) and repeat steps 2 and 3, write down input voltage  $V_2$
5. External voltage to DAC count conversion: DAC counts = (ext.voltage + 10V)\*204.8. Since DAC counts must be integer, values have to be rounded up or down.

**Recalibrate the monochromator with internal wavelength control via RS-232:**

1. Switch to internal wavelength control: send via RS-232 **SR,0!**
2. Insert filter #1 (380 nm) into the photodiode holder. Aim the light coming out of the light guide onto the photodiode.
3. Wavelength Scan: Increment analog port #1 and read the PhotoCurrent until PhotoCurrent is maximal:
 

Command	Answer	
<b>WA,1,0;</b>	WA,0!	(set analog port #1; start with 0 counts = -10V, 4095 counts = +10V)
<b>RA,1;</b>	RA, 1, 137!	(read 137 counts on PhotoCurrent channel AD-1/I)

Increment counts on analog port #1 by 4 counts each cycle until PhotoCurrent counts are maximal. Write down the counts of the analog port #1. It's the DAC1 value.
4. Insert filter #2 (505 nm) and repeat procedure 3. and write down the DAC2 value.
5. Save new calibration: **WC, 380, DAC1 value, 505, DAC2 value;**

**Reconfigure the wavelength protocol**

Establish a connection between a terminal program and the RS-232 serial port of the ADC. Send a string with the following format to the ADC

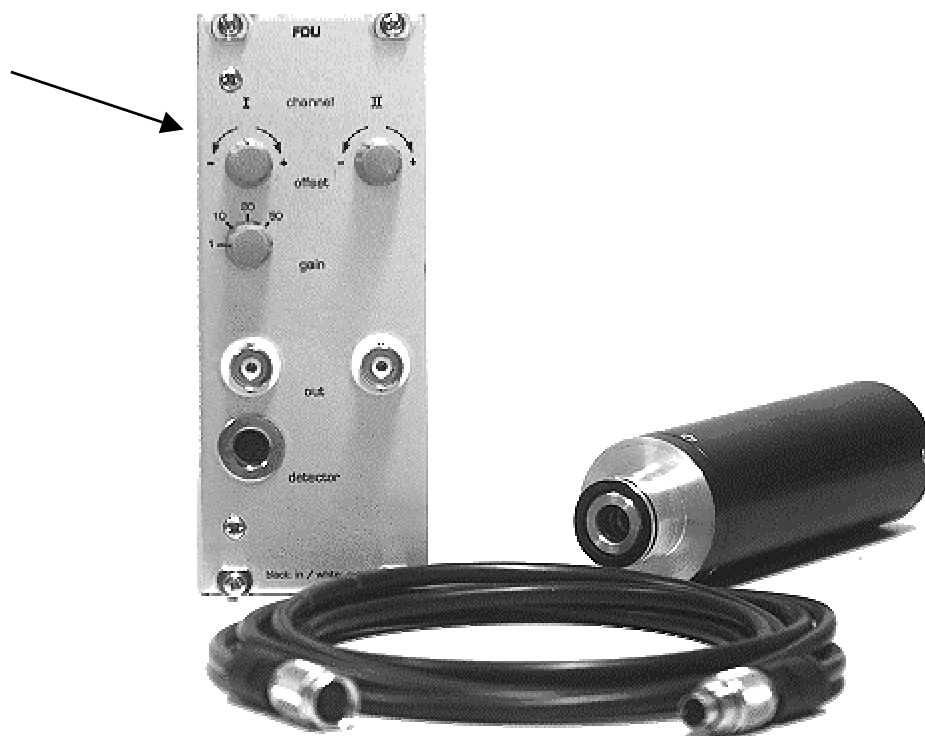
**PD, dt, it1, it2, rt, w1, w2, w3;**

- dt = dead time
- it1, it2 = integration times 1 and 2
- rt = resting time
- w1, w2, w3 = wavelength 1, 2, and 3

you must enter times in units of 100 $\mu$ s (valid values 2 .. 56000) and wavelength in nm. If ADC answer is PD,0! entry was valid: PD,! indicates a definition error, e.g. w3 (resting wavelength is below the minimal possible wavelength).

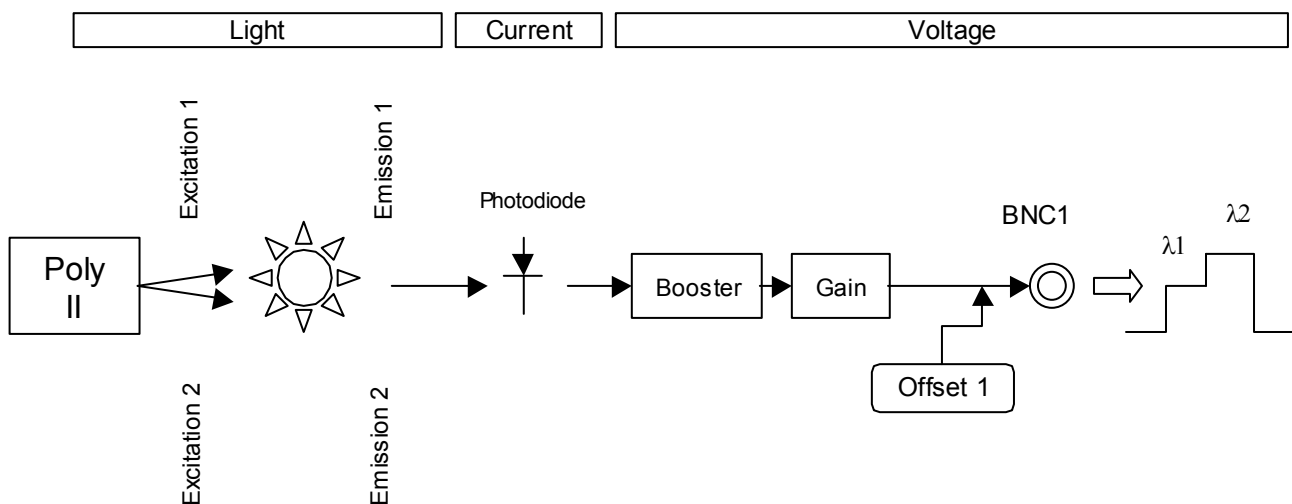
## 1.6 FDU: Fluorescence Detection unit, dual excitation / single emission

### Fluorescence Detection Unit with Photodiode detector



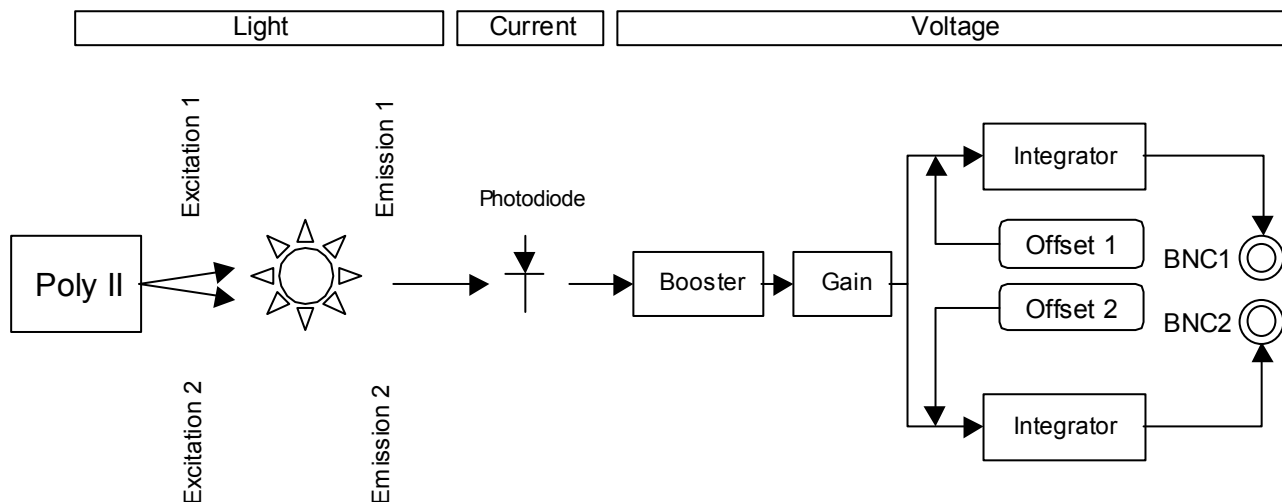
#### Signal flow in the fluorescence detection unit without integration

In most applications the system will be used in dual excitation /single emission mode. The photodiode detector head converts the photo current to a voltage signal. On the FDU board the signal passes a booster and gain circuit. A negative or positive offset voltage can be added. The left plug carries the output which is composed of the two subsequent emissions corresponding to the two excitation wavelengths (e.g. 340 and 380 nm in the case of Fura2). The software of the acquisition system (e.g. Heka Fura extensions) has to separate these emissions considering the settling time of the monochromator.



**Signal flow in the fluorescence detection unit equipped with integration**

In most applications the system will be used in dual excitation /single emission mode. The photodiode detector head converts the photo current to a voltage signal. On the FDU board the signal passes a booster and gain circuit. To the two separate channels you can add a negative or positive offset voltage. Each channel will be integrated separately under timing control of the ADC. The integrated values will be put onto a Sample & Hold circuitry and delivered for digitisation to the two BNC plugs on the front panel of the FDU board.



## 1.7 Photodiode detector head

The photodiode detector head consists of three parts:

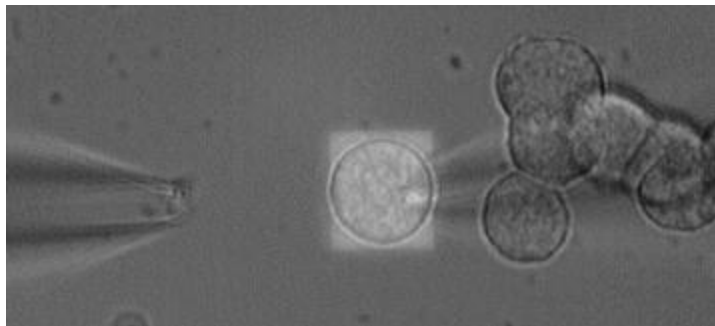
- a three lens optics that guides the fluorescence light onto the sensitive area of the photodiode
- a low noise, high sensitivity photodiode (Hamamatsu S5973-02)
- a low noise high gain current to voltage converter

### Specifications

	Condition	Min	Typ.	Max	Unit
free aperture of 3-lens optic			6.4		mm
sensitive area in object plane of the microscope			6.4/Magnification of objective		mm
diameter of sensitive area of photodiode			0.4		mm
Spectral response range			320-1000		nm
Peak sensitivity ( $\lambda_p$ )			760		nm
	$\lambda_p$	0.36	0.44		A/W
Photo Sensitivity	410nm	0.25	0.32		A/W
Quantum efficiency	410nm	75	97		%
Dark Current	VR=3.3V		0.01	0.1	nA
Capacitance			1.6		pF
current/voltage conversion			20		pA/V
Voltage output				10	V

## 1.8 Viewfinder III:

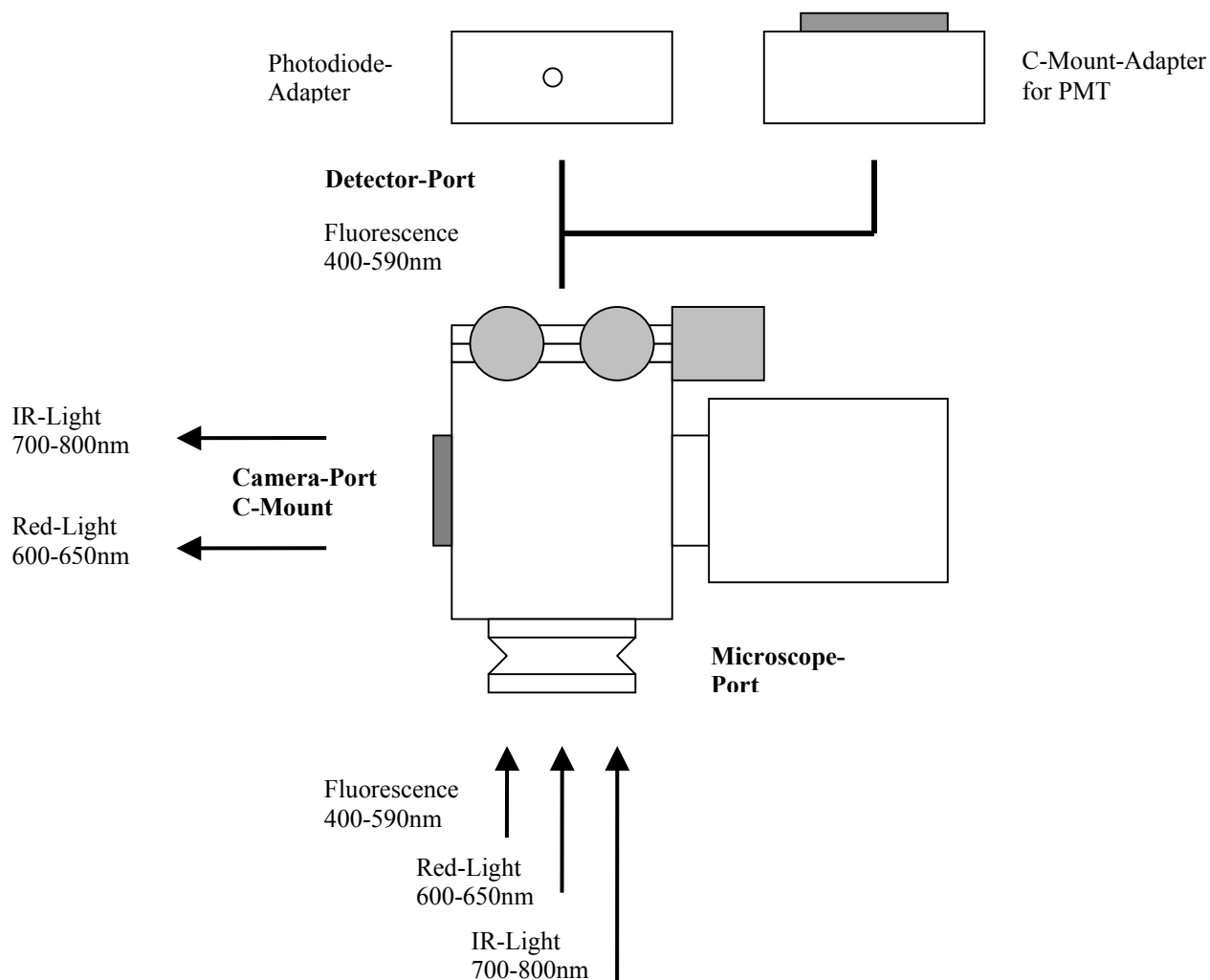
Visualise your preparation and the fluorescence detection area on a video monitor simultaneously.



### 1.8.1 Viewfinder Features

- **enhanced optics**
  - 50% red light reach the CCD
  - 80% IR light reach the CCD
  - 95% fluorescence light reach the fluorescence detector
- **mechanics**
  - C-mount adapter allows to choose between several camera options
  - new aperture focussing device
- **control of the aperture stop**
  - restrict the detection area with the rectangular aperture stop
  - adjust size and position of the aperture stop
  - do both under visual control on the video monitor
  - continuous control over the position of the aperture stop during the experiment and simultaneous fluorescence measurement
  - no mechanical movement to turn visualisation on/off
- **visualisation of the preparation**
  - times more light reaches the CCD camera compared to ViewFinder II
  - suitable for IR-DIC contrast enhancement methods

### 1.8.2 Schematic of ViewFinder III:



### 1.8.3 Installation

Attach the viewfinder by means of the attachment flange to the microscope.

Put together the cord of the external power supply and the video camera.

Connect the video cable from "video out" of the camera to the "in" plug on the backside of the monitor.

### 1.8.4 Filter K65:

This filter is a bandpass at 650nm with a HWHM of 50nm and is placed in the transmission light path of the microscope. The red light is used for two purposes: to illuminate the preparation and also the square aperture inside the viewfinder. Both is imaged onto the CCD camera of the viewfinder and displayed on the monitor. The red light is chosen since it does not make any harm to the preparation. A corresponding cut off filter (SP580) is placed in front of the photodetector(s). Therefore the red light can be left on during the fluorescence measurements for observation since it does not hit the detector but only the fluorescence. Due to the filter the fluorescence wavelength is limited to 400nm-590nm.

## 1.9 Photomultiplier detector head

The photomultiplier can be used in combination with the FDU-unit or as stand alone version which is connected directly to your data acquisition system.

### 1.9.1 Installation

Assemble the photomultiplier to the Viewfinder. If you don't use a Viewfinder connect the photomultiplier with the TV-adapter C-mount to the microscope.

The photomultiplier input ( $V_{cc}$ ) is connected via the grey cable (7 cores) to the output "head" of the photomultiplier board (PMS) in the control unit.

Connect the "signal out" plug of the photomultiplier to your data acquisition system. Please refer to the attached documentation of the photomultiplier for further information.

## 1.10 Using your photometry setup with HEKAs Fura extension

If you use the Heka software extension to *pulse* or *X-chart* the wavelength is controlled by an analogue voltage. Hence the  $\lambda$  +/- 10V plug of your control unit has to be connected to the corresponding output of your data acquisition hardware. Note: If you are using X-chart without the Fura extension you can record data but cannot control the wavelength with this program.

Depending on the type of your photometry system the  $\lambda$  +/- 10V plug is either

- in the ADC board - if you use a monochromator with integrated ADC
- in the ASC board - if you use a photometry system without ADC
- in the ISC board - if you use a combined photometry/imaging system
- in the ADC board of the separate control unit - if you use a combined photometry/imaging system in a configuration with separate control unit

Please follow the instructions in the manual from HEKA for calibration of the monochromator and for informations about the software.

Use the supplied I/V converter to connect the photodiode of the calibration kit to the data acquisition interface when you perform a calibration. Depending on the position of the switch a 100 or 1000 Ohm resistor is in parallel to the photodiode. In position 0 the photodiode is directly connected to the output for measurement of photocurrent e.g. with the multimeter supplied (see also adjustment of the lamp).