



# ***pc1***

Photon Counting Steady-State Spectrofluorometer

*Hardware Manual*

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## I. Quick Start Up and Logon

### 1. Daily Operation

#### 1.1 Introduction

For daily operation of the ISS-PC1 steady-state Spectrofluorometer a couple important aspects have to be kept in mind. Chapter one guides you through a quick check of the instrument configuration and readies it for normal everyday use.

Subsequent chapters in sections I to III of this manual will guide the new or experienced user, who wants to make a serious commitment to understand and apply the principles of optical spectroscopy, in a step-by-step fashion through the process of understanding this state-of-the-art instrument, which has been specifically designed for research purposes with great flexibility in mind. Optical spectroscopy covers a very broad array of measurement conditions.

With patience you will master its use for a multitude of experiments. Help is always available.

You will immediately notice that, with respect to the previous manual, this manual details first the daily operation followed by an in-depth description of all available functions. Technical aspects of installation and maintenance of the instrument have moved to sections IV through VI of this manual.



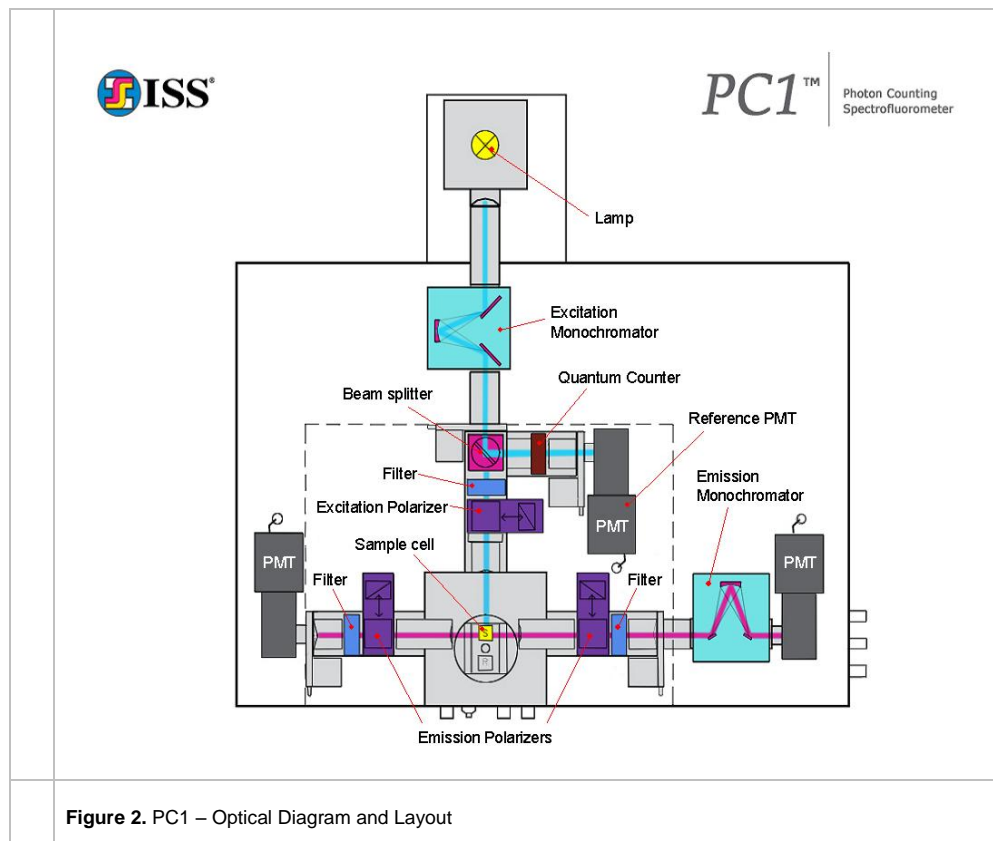


Figure 2. PC1 – Optical Diagram and Layout

## 1.2 Startup / Shutdown Procedure

- Please turn your computer and instrument off. Turn the current control knob of the lamp power supply completely counter-clock-wise (CCW). Switch the lamp power supply on.

For best intensity stability set the current control knob to 18A. Use at least a 1-hour warm-up period for lamp and instrument electronics. Use 2 mm excitation slits for maximum intensity at the sample. Check that the lever of the iris diaphragm behind the turret points to the left (open position).
- Turn computer, ISSPC instrument and printer / plotter on.

The instrument ON switch is located on the right rear side panel. The PMT house LED's turn on with the PMT house switch set to manual. Check the voltage potentiometer setting is 10.0 (for photon-counting mode of operation).
- Click the ISS icon or type cd\ISS and press <Enter> (cd ==> change directory)  
Type ISS and press <Enter> to start the ISSPC program.

Press Enter> to pass the Logo.  
Press <1> or <Enter> to start the software.

4. Check that dark signals (counts/sec) appear. An example for a standard PC1:

**Photon-Counting Mode of Operation** (Signals displayed in Counts/sec.):

SAM: 453	REF: 75	AUX: 480	TB: 1.0	Exc:400	Em:500
			(sec.)	(nm)	(nm)
<i>Mon. Side</i>		<i>Filter. Side</i>	<i>TimeBase</i>		
<i>Sample</i>	<i>Reference</i>	<i>Auxiliary channel</i>			
<i>Right em.</i>	<i>Reference</i>	<i>Left em. Detectors</i>			

**Analog Mode of Operation** (Signals displayed in mV):

SAM: -1.2	REF: 0.14	AUX:	TB: 1.0	Exc:400	Em:500
<i>Sample</i>	<i>Reference</i>	<i>Auxiliary channel</i>			

5. Bypass this step when on start-up, the program asked you to verify the monochromator dial wavelengths. You can double-check the settings by pressing <F8> to enter the Instrument Status screen. When required press <F4>, read the excitation monochromator wavelength dial. Enter the value in the appropriate window and press <Enter>. Use the same procedure for the emission monochromator(s).

Selection of data storage directory and filename.

- 6a. First time user or creation of a new datafile or directory:  
Press <5> Disk Utilities in the Main Spectra Menu and <4> Create Data File. For example enter in the Name: window: C:\DATA\MYDATA1 and press <F10> to create Mydata1.cat and .dat. This creates a filename and stores the chosen name on hard or floppy disk in a directory of your choice.
- 6b. Select an already existing (sub)directory and filename.  
Press <5> Disk Utilities in the Main Spectra Menu. Press <5> Open Primary File. Use Tab key and arrow Up / Down to make your choice. Press <F10>.

Press <F6> to check the Directory of the File: C:\DATA\MYDATA1.  
For a newly created filename one sees a black screen: No spectra have been taken yet.

The titles of up to 160 spectra are stored in Mydata1.cat. This is the maximum number of spectra that can be stored in a single Filename. The spectral data themselves are stored in the associated file Mydata.dat.

7. Check the Reference detector intensity for proper Signal-to-Noise (S/N) ratio. The signal should not overload detector or photon-counting electronics (a good range: counts/sec. < 500,000) nor should the signal be too small (counts/sec < {100+dark} ). The standard instrument configuration and mode of operation is photon-counting.

	<b>SAM</b>	<b>REF</b>	<b>AUX</b>
	<i>Monochromator</i>	<i>Monitor Detector</i>	<i>Filter Side</i>
Signal from	Right Emission	Reference	Left emission

Signal Level	<5,000,000	1000< 1 <500,000	<5,000,000
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**Table 1.2-1 Suggested Photon-Counting Signal Levels**

Adjust reference detector intensity as required, by using Neutral Density filters (Photon-counting mode of operation).

8. Prepare Sample cuvette solution to take a spectrum  
 Select <1> Acquire Data from the main Spectra Menu. Then select <1> Take a Spectrum. Enter your choices. Check also the <Alt-A> advanced options for printer, temperature, spectral bandwidth options etc. Press <F10> to start the measurement. Check that the dark signal is measured with the excitation shutter closed.  
  
 A *beep* signals the end of the measurement. Edit spectrum title when necessary. Press <F10> to store the spectrum in binary format. See 10. below for conversion to ASCII format.  
  
 <F6> allows a check of stored spectra titles in the directory of the Mydata1.cat file.  
  
 To improve Sample detector Signal-to-Noise, increase the integration time per wavelength step. Press <F9> to change the TimeBase (TB).
  9. Displaying the spectral data: Several Possibilities.
    - a) Press <F5> to view the data in memory.
    - b) Use <F6>, enter spectrum number or use Arrow Up / Down keys and Press V(iew) or
    - c) Press <Esc> to exit the Acquire Menu. Press <2>. Display/Print/Plot Spectra.

Press <2> Display Records.  
 Use <5> High Resolution Graphics to overlap spectra.

Press <F7> for Help in this screen. <F1> shows the directory etc., <F4> shows composition. Press <F5> to set the Plot parameters. Press <F10> to create a plot. To add text to the graph, see Chapter 9 of this manual.
  10. Exporting ASCII data.  
 Press <5> Disk Utilities in the main Spectra Menu. Press <2> Export Spectra Data to ASCII. Select a spectrum number targeted for conversion. Press <F10>. Enter in the ASCII File Window: A:\spectr1.asc or A:\spectr1.txt for example to store converted data directly on a floppy disk. The binary spectra data are converted to ASCII values and stored in the C:\DATA directory.
  11. Instrument Shut down.  
 Press <Esc> and Exit the ISSPC program <Y>. <Esc> exits the ISS Shell program. Set the lamp current back to 10A. Let the lamp cool down for several minutes.  
 Copy your data to a floppy: c:\data>copy Mydata1.\* b: for safe keeping.
  12. Please write down operator, number of hours of instrument use and lamp current in the instrument log book.
- Note: Chapter 4 of this manual provides you with a tutorial giving general information on the best route to get proper spectra.

Customer Support:

*Please feel free to contact ISS customer support at Telephone (217)-359-8681 ,  
Fax (217)-359-7879 or e-mail [service@iss.com](mailto:service@iss.com) for help.*

## 2. Instrument Specifications

### Measurements (steady-state fluorescence)

Corrected excitation and emission spectra  
Excitation-emission matrices  
Polarization (anisotropy) measurements  
Intracellular Ca<sup>+</sup> measurements  
Millisecond kinetics in photon counting mode  
Dual wavelength excitation or emission measurements

### Instrument Specifications

#### Light Sources

- 300 W high-pressure xenon arc lamp, 45 mW/nm brightness at 275 nm
- Lamp power supply: controllable in current, with time meter

#### Optional Sources

- laser diodes
- light emitting diodes (LEDs)
- continuous wave lasers (argon-ion, krypton-ion, helium-cadmium, etc.)

#### Monochromators

- single concave aberration corrected holographic grating\
- optional: double grating
- focal length: 100 mm or 200 mm
- linear dispersion: 8 nm/mm or 4 nm/mm (single grating)
- stray light:  $10^{-5}$  outside the bandpass of the HeNe line
- wavelength range: from 200 nm to 1200 nm (depending upon the type of grating selected)
- wavelength accuracy: +/- 0.2 nm
- wavelength reproducibility: +/- 0.25 nm
- slew rate: 160 nm/s

#### Optics

##### Lenses

- UV-grade fused silica lenses

##### Polarizers

- UV grade Glan-Thompson, 10x10 mm, L/A=2.0
- UV grade Glan-Thompson, 14x14 mm, L/A=2.0
- UV grade Glan-Taylor, 10x10 mm, L/A=2.0 (for high power lasers)

##### Optical design and collection geometry

- parallel beam design for precise polarization measurements
- T-format for simultaneous acquisition on 2 emission channels

#### Detectors

- side-on photomultiplier tubes in room-temperature or cooled housing
- optional: CCD camera
- emission channels PMTs: Model R928P by Hamamatsu
- reference detector PMT: Model R928 by Hamamatsu
- PMT wavelength range: 240-900 nm

**Detection Modes**

- photon counting electronics, 10 KHz, on 3 independent channels
- optional: Analog Output

**Pre-amplifier Discriminators**

- 80 MHz bandwidth, TTL output

**Dynamic Range**

- linear up to 4 million counts/second

**Sensitivity**

- 800 fM of fluorescein (with cooled PMT housing)

**Signal-to-Noise ratio**

- 2000:1 (room temperature PMT)
- 6000:1 (cooled PMT)

**Automation**

- control of 4 shutters
- up to 3 monochromators
- 3 polarizers
- sample holder rotation
- stirrers
- filterwheel

**Instrument Interface to RS232 devices**

- titrators
- stopped-flow apparatus
- Peltier sample compartment
- flow-through temperature bath

**Computer and Operating System**

- Intel-type CPU, Windows XP operating system

**Power Requirements**

- universal power input: 110-240 V, 50/60 Hz, 400 VAC

**Optical Bench**

- dimensions: 885mm (L) x 600mm (W) x 330mm (H)
- dimensions with the lamp: 885mm (L) x 835mm (W) x 330mm (H)
- weight: 40 Kg

### **3. Warranty**

#### **A. General Conditions**

All ISS manufactured instruments are warranted against defective materials and workmanship for one year from the date of shipment. The instruments must be used for the function they have been designed for, as described in the instruction manual. A Return Material Authorization (RMA) number is required before returning any instrument to the ISS factory for repairs.

Should this product malfunction during the warranty period, ISS will, at its option, repair or replace it at no charge, provided that the products have not been subjected to misuse, abuse, or unauthorized alterations, modifications, and/or repairs.

All expressed and implied warranties for this product include, but are not limited to, the warranties of merchantability and fitness for a particular purpose, are limited in duration to the above one year period. Some states do not allow limitations on how long an implied warranty lasts, so the above limitations may not apply to you.

Under no circumstances will ISS Inc. be liable in any way to the user for damages, including any lost profits, lost savings, or other incidental or consequential damages arising out of the use, or the inability to use, such products.

#### **B. Expired Warranty**

ISS will repair instruments with expired warranty at the current part and labor prices.

#### **C. Non-ISS Parts**

Although ISS Inc. may frequently supply, as part of a system, equipment manufactured by other companies, the only warranty that shall apply to such equipment is the warranty offered by the original manufacturer.

#### **D. Field Service**

ISS Inc. will substitute free of charge for parts and labor all defective parts during the one year warranty period, though travel and local expenses will be paid by the customer.

#### **E. Transportation Damage**

Packages should be carefully examined upon receipt for evidence of damage caused by shipping. If damage is found, notify ISS Ins. immediately. Preserve all packages, cartons and documents.

### **4. Fluorescence Spectroscopy**

Fluorescence detection is a very sensitive but unfortunately also very sensitive to impurities, either coming from solvents or buffers that are used or from improper cuvette handling. It is good practice to make control experiments and check the emission properties of each and every component used in an experiment. If one component shows substantial fluorescence, background fluorescence subtraction methods provided in the software could be applied to correct these. A similar correction may be required to eliminate the influence of scattered light, elastic (Rayleigh, first or second order) or inelastic (Raman scatter from water) from the recorded spectra. Elimination of scattered light and the selection of proper excitation and emission filters is even more critical for polarization / anisotropy experiments.

All of the above aspects are pointed out to the user during the instruction following instrument installation. Nevertheless every user of this instrument should familiarize her / himself with the following general aspects relating to optical spectroscopy in the ultra violet (UV), visible (VIS) and Near Infra Red (NIR) region of the spectrum in order to get the best results.

Examples of Spectral Data: Emission and excitation spectra and a time scan.

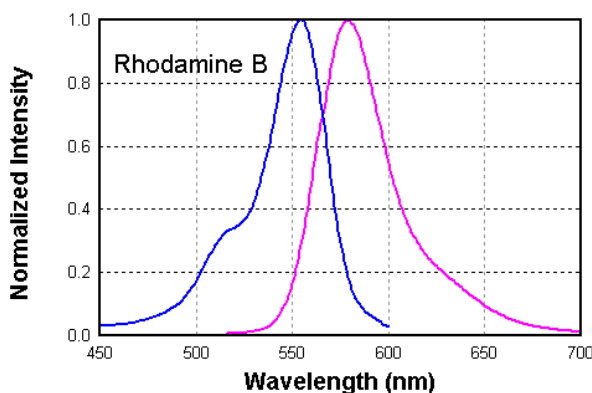


Figure 0-1 Excitation (blue): Emission Wavelength is fixed and Emission Spectrum (red): Excitation Wavelength is fixed

## 4.1 Experiments

This section describes the procedures of acquiring fluorescence data with the PC1 instrument. Also described in detail is how to configure the instrument for a specific measurement.

### 4.1.1. Instrument Preparation

Please follow the Daily Operations in Section I, Chapter 1.

Check that the reference detector shutter is open (Lever points 45 degrees up).

Check proper signal levels on the reference detector (about 10,000 to 500,000 counts/sec.).

### 4.1.2. Cuvettes

#### Selection of Cuvettes: Plastic, Glass or Quartz

For fluorescence measurements in the visible and near-infrared region plastic or glass cuvettes should suffice and they are less expensive than quartz cuvettes. However, plastic cuvettes may not be good for polarization measurements as the cuvette material may prevent to obtain proper

polarization data. It is advised to compare the results obtained in a plastic cuvette with those from a glass cuvette. For excitation wavelengths below 350 nm good quality quartz cuvettes with lowest fluorescence background should be selected. Such measurements include measurements of DNA and proteins. Deep UV transmitting cuvettes (Suprasil / Herasil) are available to minimize luminescence background from the cuvettes.

A quick test can be performed to check what type of cuvette material is at hand: glass or quartz. A white business card is placed in 280 nm excitation light. The paper fluorescence appears bright blue to the eye. Now place a cuvette of unknown material in the optical path, between the business card and the excitation monochromator. When the intensity of the business card fluorescence does not noticeably diminish the cuvette is made out of UV transmitting quartz or fused silica material.

For low temperature, LN<sub>2</sub>, measurements special thick-walled cuvettes have to be used. Standard, room temperature, cuvettes can not withstand the stresses caused by expanding liquid.

### Cuvette Size and Shape Selection

When large sample quantities are readily available, 10 x 10 mm optical path cuvettes can be used. Outside dimensions for this type of cuvette are ½ x ½ inch.

For smaller sample volumes 10 x 3, 10 x 2, 10 x 1, 5 x 5 or 3 x 3 mm optical path cuvettes can be selected. Thick walled cuvettes may scatter excitation light more due to the extra invisible microscopic inclusions like air bubbles. Special holders are available or can be made to order. These special holders have observation windows that prevent illumination of the cuvette walls, which may otherwise generate a large scatter background signal.

Beware of the placement of the 10 x 2 mm cuvette in the optical path. At times a 10x2 mm cuvette may be inserted with the 10 mm optical path pointing to the excitation source. With the variable iris opening full open, the excitation light may hit the cuvette walls. This will generate a large amount of scattered light. This can be particularly problematic for polarization measurements.

Polarization measurements may not give a correct answer when the focused excitation light scatters from the cuvette walls. The arrow in the figure below indicates the direction of the incoming excitation beam.

Highly scattering samples can be placed in 10 x 1 mm cuvettes or in front surface triangular cuvettes, although the 45 degree angle of the front face will scatter all excitation light into the detector.

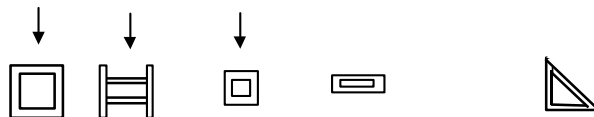
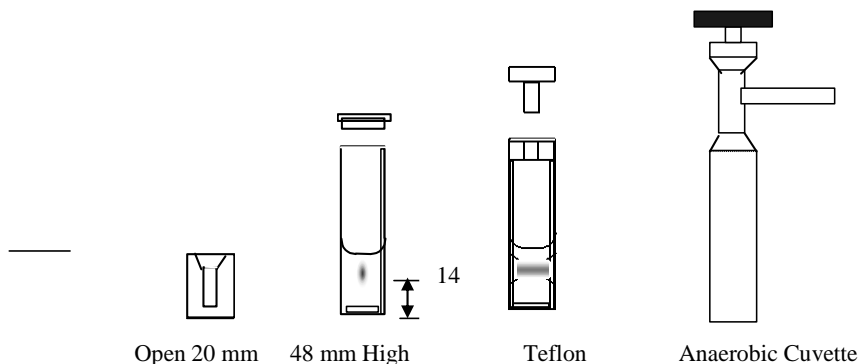


Figure 4

The optical path of the excitation light is centered 14 mm above the bottom of the cuvette. The meniscus of the cuvette solution should well clear the excitation volume. Inserted magnetic stirrers, preferentially Teflon coated, should also not obstruct the illumination beam. The minimum filling volume is about 1.5 ml.



**Figure 4.1-2 Side View of Commercially Available Cuvettes**

For anaerobic work, stop cock cuvettes are recommended. They can be closed after filling the cuvette with Nitrogen gas for example. These cuvettes keep their controlled atmosphere for many hours to days. A sample compartment extension is available.

Square Teflon caps or Teflon stoppers are commercially available. They prevent dust or bacteria from entering the solution.

### Cuvette Preparation and Storage

Clean a cuvette when necessary. Use soft cotton Q-tips, hot water and soap or a cuvette cleaner. Rinse thoroughly. Quartz and glass cuvettes when not in use are best stored overnight in 10-20% nitric acid. Before use, rinse the cuvettes with distilled water, buffer or other appropriate fluid. More information can be found in the tests section about expected water quality necessary to perform proper background-free experiments.

### Cuvette Handling

Never handle the cuvettes in such a way that fingerprints contaminate the outside cuvette walls. The optical axis for your instrument is 14 mm above the bottom of the cuvette. Certainly do not touch this area. Fingerprint fluorescence can create a huge luminescence background. Just think of its application in the forensic area. Work above a table surface, to prevent damage in case the cuvette drops. Place an open rubber mat in the sink and on the floor in front of the instrument.

### WARNING



**NEVER FILL A TEFLON STOPPERED CUVETTE TO THE RIM WITH NO AIRSPACE LEFT. YOU MAY WELL BLOW OUT A CUVETTE WALL.**

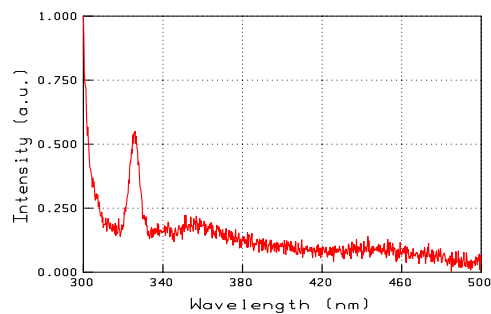
Liquid has a very low compressibility and pressing the Teflon stopper in the cuvette will blow out a cuvette wall. This is also true for the triangular quantum counter cuvette.

#### 4.1.3. Handling of Chemicals

Apart from the precautions on the label, material stored in a cold room or freezer should be warmed up before opening the bottle. Otherwise air moisture will collect in the opened bottle.

#### 4.1.4. Buffer Solutions and Background Fluorescence

Buffer solutions stored in a refrigerator should be warmed-up, before they are used in room temperature experiments. Polarization measurements are very sensitive for solvent viscosity. Check unknown buffer solutions for hindering fluorescence background. Automatic background subtraction procedures are provided in the software and can be used to eliminate the contribution of the buffer solution to the measured emission intensity. Avoid dust particles in the solution. Do NOT store water or buffer in plastic bottles unless it is clear from experiments that no emission background is generated by plasticizers leaching into the solvent over time. Parafilm will dissolve in several solvents. When samples only weakly emit (relative to the Raman line of water or the buffer fluorescence) external fluorophores could be used or select another buffer.



**Figure 4.1-3 Example of a Clean Buffer Solution: MOPS pH 7.3**

The Raman line of water is clearly visible above the low background. Some buffers show a very large background fluorescence.

#### 4.1.5. Transparent, Liquid Samples

##### Filling a Cuvette, Magnetic Stirring and Small Sample Volumes

On occasion the solvent meniscus is so low that the excitation beam, centered at 14 mm from the bottom of the cuvette, hits it. Use a dentist mirror to check this and prop the cuvette up or use a smaller cuvette size. Another obstruction can be formed by oversized magnetic stirrers or even small stirring bars when the cuvette is propped up.

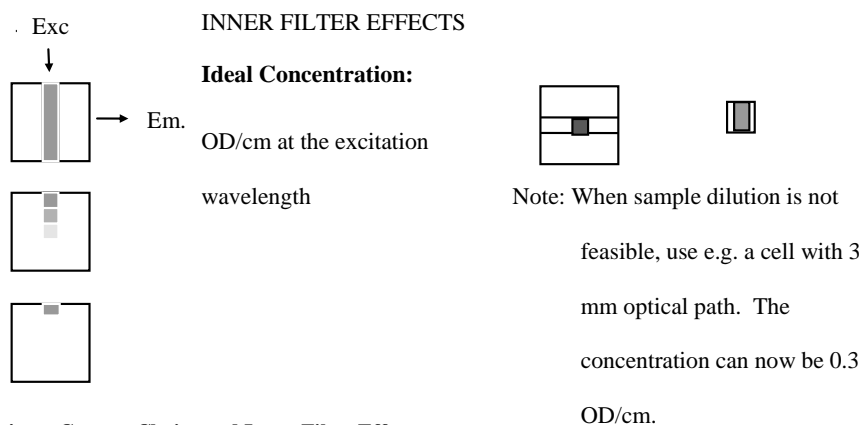


Figure 4.1-4 Cuvette Choice and Inner Filter Effects

##### Optical Density

Prepare a sample with an absorption at the excitation wavelength equal to an O.D. (Optical Density) of 0.05 to maximally 0.1/cm (Lambert-Beer law). Use a Spectrophotometer if necessary. One may be able to see the fluorescence by eye. Hold the beam in the light beam. Check that the observed fluorescence is homogeneously emitted by the illuminated excitation volume. This avoids spectral distortion due to inner filter effects.

#### 4.1.6. Solid Samples

##### Transparent Solid Samples

They should be positioned in available solid sample holders in such a way that the reflected light points away from the emission detector. Special holders are available that eliminate the possibility of detecting a background from the black anodized surface.

Komentář [Mv1]:

##### Opaque and Highly Scattering Samples

These opaque samples can be positioned such that the normal to the surface makes a 30 or 60 degree angle with respect to the incoming light. 45 degree insertion angles should be avoided, since the detector may receive very large amounts of reflected / scattered excitation light. For these samples front-surface accessories are available.

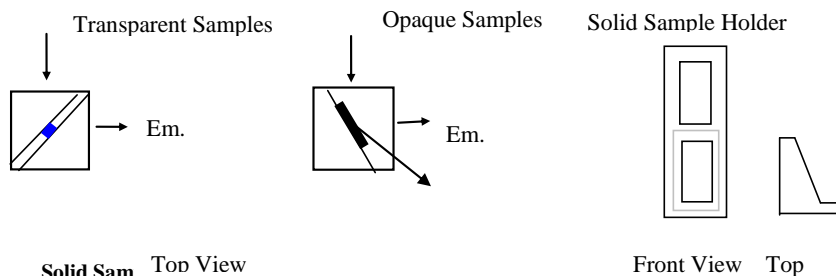


Figure 4.1-5 Solid Sam<sub>u</sub> Top View

Front View Top

#### 4.1.7. Sample Stability and Time-Dependent Phenomena

Once samples have been prepared they may exhibit a variety of time dependent effects. One should be well aware of these effects.

Process	Time-Dependent Changes in Sample Intensity / Polarization
Aggregation	The observed signal decreases since part of the sample is not kept afloat by Brownian motion and settles on the bottom of the cuvette
Attachment to cuvette walls	Dilution by many orders of magnitude still gives a nice signal
Bleaching	Dissolved molecules suffer photon damage and are destroyed
Cuvette dirty	Rhodamine release into newly prepared sample
Fusion effects	Vesicles aggregate from 50 to 100 to 200 um over time. Vesicle curvature changes, influencing the observed fluorescence
Folding / Unfolding / Denaturation	Intensity changes occur since a buried or exposed Tryptophan residue in a protein has a different quantum efficiency
Solvent evaporation	Sample concentration increases while ethanol evaporates
Solvent temperature	Samples retrieved from the cold room warm up and show a changing fluorescence emission
Undissolved powder	Example: p-terphenyl in ethanol has not completely dissolved. Minute p-terphenyl particles continue dissolving continuously increasing the fluorescence. The particles while floating through the beam also increase the intensity noise.

Table 4.1-1 Undesirable Time-Dependent Effects

#### 4.1.8. Selection of the Spectral Observation Window

In choosing a method of wavelength selection for a particular experiment, you should have an idea about the general optical properties of the sample under study. Solutions usually exhibit a 50 – 100 nm wide emission spectrum, contrary to powdery samples, which often display 0.1-5 nm narrow spikes. When the sample is bright enough any spectral selection device can be used. Otherwise a trade-off may exist between required minimum intensity for a proper Signal-to-Noise ratio and the desired spectral resolution.

Wavelength Selecting Device	Observed Intensity	Transmitted Spectral Region	Spectral Selection
Absorption type Filter	High	High pass	Poor
		Wide Band	Better
		Low pass	Poor
Interference Filter	Medium	Medium	Better
Monochromator	Low	Small	Still Better
Double Monochromator	Even lower	Even Smaller	Even Better

**Table 4.1-2 Wavelength Selecting Devices for Optical Spectroscopy**

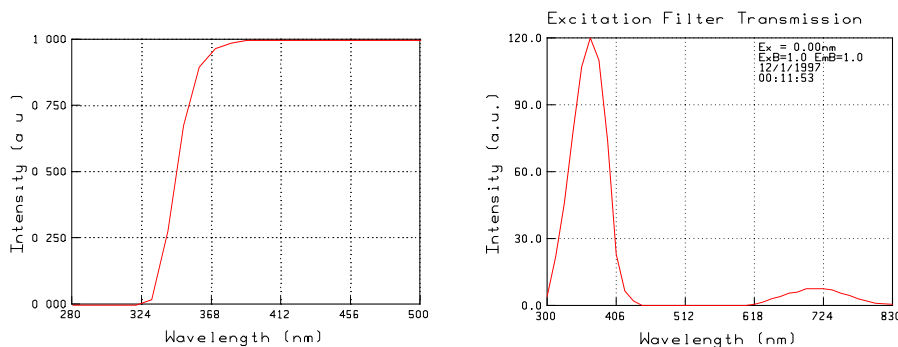
Glass or quartz substrate (for UV transmission) low and high pass filters are economical devices to pass or block excitation or emission light. Their transmission is usually very high (90% or better) beyond a certain wavelength. Wavelength discrimination is not as good as with interference filters or monochromators. Filters come in convenient shapes: e.g. two (2) inch square or 1 inch round.

Optical filters can create undesired emission: filters may show autoluminescence or transmit outside transmission bands as specified by the manufacturer. Always check this. Measure over the complete spectral region from UV to NIR the transmission of every filter used.

At times even a monochromator is not sufficient and additional optical filters have to be used. This happens, for example, when second order excitation or emission peaks have to be removed.

**Absorption Type Optical Filters** Typically this type of colored glass optical filter is classified with the wavelength for their 50% transmission intensity. Transmission of these filters increases from 0% to 95% in a sigmoid curve over a wavelength range of 40-60 nm.

Other filters may exhibit several bands in which they transmit. Without additional filters unwanted light (second order excitation light for example) may reach the sample. This can be rather interesting when polarization data are taken and you are not aware of this effect.



**Figure 4.1-6** Transmission Curves for a Schott WG-345 (Left) and for a Corning 3-75 Optical Filter (Right)

#### Polarization Effects in Absorption Type Optical Filters

Transmission properties for tilted optical filters illuminated by vertical and horizontally polarized light are different, Fresnel and Brewster effects. Therefore keep them straight up and vertically positioned in the supplied filter holders.

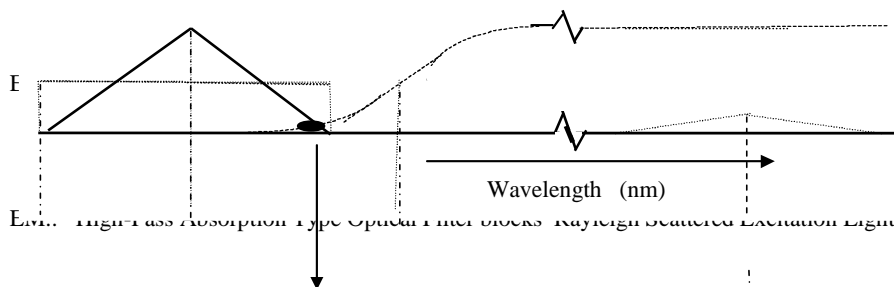
#### Low pass Filter: Elimination of Second Order Excitation Light

This type of filter is commonly used to prevent second order excitation light from reaching the emission channel detectors. See the figure below. The region around 600 nm (2 x 300 nm exc. wavelength) shows the conspicuous presence of this 2<sup>nd</sup> order.

#### High Pass, Absorption Type, Filter

These filters may show autoluminescence. Check this by holding the filter in the excitation beam. Observe the intensity of the sideways emerging autoluminescence. Select a filter with the lowest perceptible amount. Recorded spectra of weakly emitting, highly scattering samples may contain a substantial amount of this autoluminescence signal. To remedy this, obtain filters with the lowest background, maximize the distance between sample and filter, increase the fluorophore concentration of the sample or use an interference filter. Close to a hot, Infra-Red heat-emitting light source like a lamp an extra Infrared blocking filter should be placed between lamp and filter.

Lamp Lightsource , Excitation Monochromator with 2.0 mm slits (16 nm FWHM bandwidth)



(but not perfectly).

Excitation **Slit Profile** at 300 nm      WG335 emission filter has a 50% Transmission at  
335 nm  
2 mm Exc. slits, FWHM 16 nm

*Overlapping region between*

*Excitation profile and filter transmission curve*      600 nm

e.g. Corruption of Polarization Data      Origin: **2nd order of 300 nm**

**exc light**

in exc. and / or em.

monochromator

Interference filter in excitation optical path:

Elimination of 2nd order exc. light entering the sample cuvette.

A low pass absorption type filter can also be used in the excitation path.

**Figure 4.1-7 Choosing Low Pass and High Pass Excitation and Emission Filters**

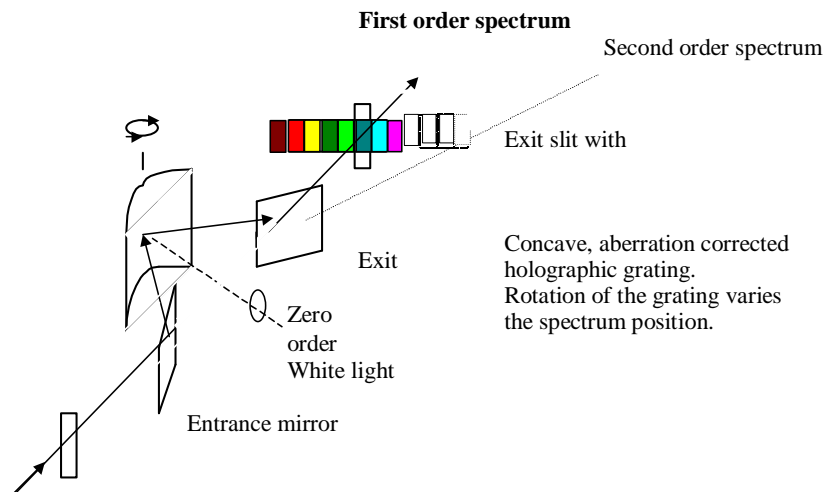
#### 4.1.9. Interference Filters: Bandpass Filters

Interference filters generally possess a better transmission than monochromators over the rather small bandpass they have been designed for. However, peak transmission for UV transmitting interference filters often drops to below 20 per cent unless specially designed. Typically, commercially available bandpass values are 5, 10, 20 and 30- 50 nm. When tilted, the transmitted center wavelength changes and transmission properties for polarized light change. In the excitation channel they are used to eliminate 2<sup>nd</sup> order light and possibly plasma lines emitted by laser light sources.

When you set the excitation monochromator to zero order an interference filter in the excitation beam may transmit more light. On the emission side a higher transmission can be obtained as compared with a monochromator. Close to a hot lamp the shiny side of the interference filter should face the light source.

#### 4.1.10. Monochromators

Monochromators (mono - chroma, one-color: Greek) provide the best wavelength selection and a lower transmitted intensity when compared with just described filters. Monochromators have the best wavelength selectivity dependent upon the chosen grating. They exhibit a wavelength dependent throughput, which is also polarization dependent. In addition higher order diffracted light is generated and may pass to the sample.



**Figure 4.1-8 Monochromator with Concave, Aberration Corrected Holographic Gratings**

A monochromator accepts incoming light and disperses it into the various colors of the spectrum. Rotation of the grating selects the color passing through the exit slit. The optics, grating and mirrors, image the entrance slit into the exit slit. Coupling optics, lenses, should not overfill the grating. This would increase the amount of scattered light inside the monochromator. Underfilling the grating lowers the designed spectral resolution. The slit height is 8 mm. Various widths for the slits are available. The installed monochromators can be removed and accurately remounted when the experiment so requires.

#### **Grating Blaze Wavelength**

By design the manufacturer can shift the peak spectral intensity throughput by adjusting the blaze angle for the grating. Gratings in the excitation channel are typically blazed at 250 nm to optimize UV excitation intensity. The emission grating is blazed at 450 nm or has a sensitivity optimized for a spectral region stretching from 200 - 800 nm. Gratings produced with holographic methods with 1200 grooves/mm providing very low straylight and ghost-free spectra.

#### **Aberration Corrected, Holographic, Concave Gratings**

Spectral impurities, ghost lines, are minimized by the consistent use of holographic gratings. Intensity throughput is optimized by the concave shape of the gratings: they act as dispersive as well as focusing elements. This reduces the use of extra focusing lenses inside the monochromator.

#### **Wavelength Indicating Dial**

The wavelength (nm) passed through the slits can be read from the dials.

#### **Polarization Bias and g(rating) Factors**

Transmission properties for monochromators illuminated by vertical or horizontally polarized light are different. This is mainly caused by the grating and to some extent by other optical elements like mirrors inside the monochromator. This effect can be corrected by selecting in the software: i.e. Enter g-factor yourself : N, i.e. measure the "g-factor".

You can activate automatic correction of wavelength and slit width dependent transmitted intensity and polarization.

#### *T-format measurements*

Make the optical paths for T-format polarization measurements as equal as possible, to keep g small. When you have an instrument with 3 detectors, you may place a neutral density filter with a proper emission filter in front of the emission monochromator set to zero-order "0" nm and equipped with 4 mm emission slits.

#### *Number of Monochromators in Use*

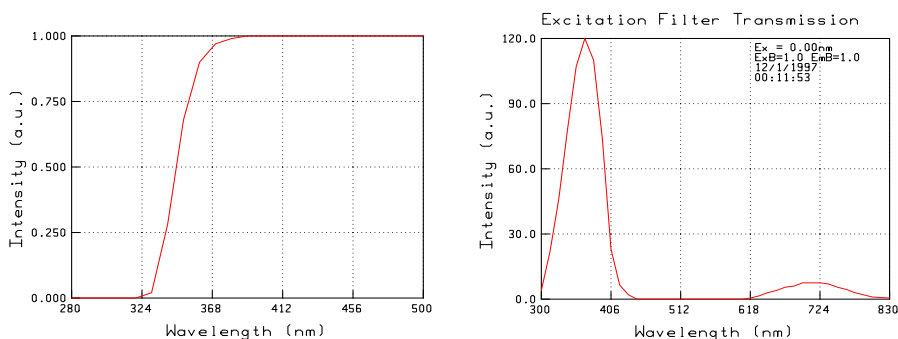
The flexibility built in to the instrument allows you to remove a monochromator temporarily to measure left and right intensities through an emission filter. You can also install a second emission monochromator on the left side of the instrument when detection through monochromators is desirable.

#### **Elimination of Second or Higher Order Light: Order Filters**

Monochromators create higher order diffracted light. Proper excitation and emission filter selection will reduce or better eliminate their influence on the collected data. This phenomenon is readily observed for weakly emitting, highly quenched samples and also with highly scattering samples like vesicles, liposomes or powders, plastic or biological fibers.

Higher order excitation light passes through the excitation monochromator. Use an interference or absorption-type low-pass blocking filter.

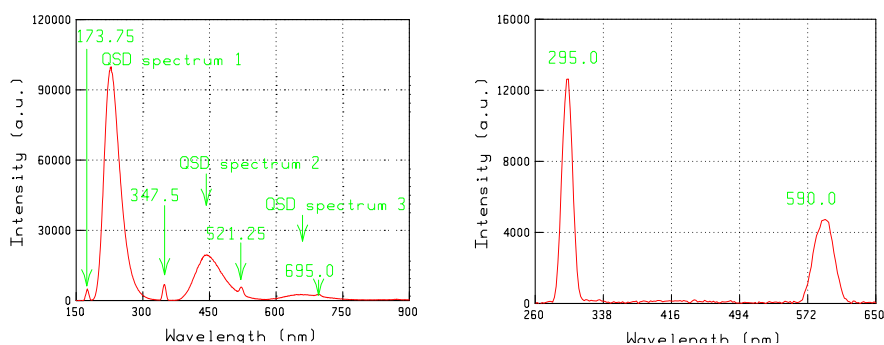
Use a blocking filter to prevent higher order scattered excitation light from passing through the emission monochromator when necessary.



**Figure 4.1-9** Highpass Absorption Type Emission Filter (WG-345) (Left); Corning 3-75 emission filter showing two (2) passbands (Right)

An instructive example is shown in the following figure. For a 600 gr/mm grating a Quinine Sulphate Dihydrate (QSD) emission spectrum was collected. (The wavelength setting of the dial of such a monochromator grating has to be multiplied by two to be comparable with the dial readings of a standard 1200 gr/mm grating).

Several orders show up in the spectral scan: 3 spectra and 3 Rayleigh scattered excitation slit profiles. Next to this figure you see a more often encountered situation: an excitation scan at 295 nm, will give a second order peak at  $2 \times 295$  nm: 590 nm. Care has been taken with additional neutral density filters in the excitation path not to saturate the emission detector.



**Figure 4.1-10** Higher Order Spectra: Quinine Sulphate Dihydrate Spectra (Left) and Rayleigh Scattered Excitation Light Using a Very Dilute Glycogen Solution (Right)

Eliminate these higher orders by inserting order blocking filters in excitation and / or emission channels.

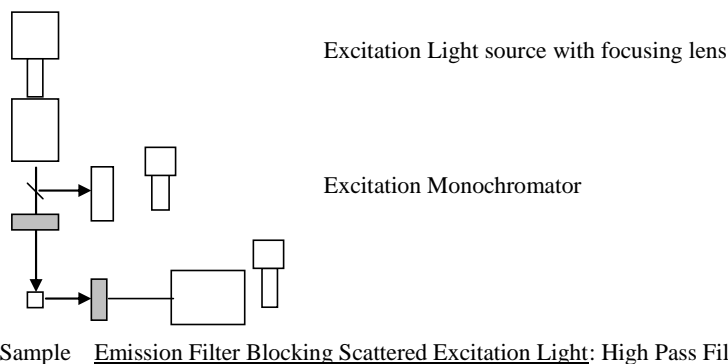


Figure 4.1-11 Optical Filter Selection for Highly Scattering Samples

#### 4.1.11. Slit width Selection for Excitation and Emission Monochromator

In selecting a suitable slit width one has to realize that with 2 equally wide slits installed the convoluted image profile is approximately a triangle and to a better approximation a Gaussian shaped profile.

The Full Width at Half Maximum (FWHM) describes the effective line profile for an imaginary rectangular profile with the same area as the approximately triangular slit profile. Slits can be exchanged to alter excitation intensity and spectral resolution. The smaller the slit the less light passes but the better the spectral resolution.

#### Slit Use and Spectral Line Profile

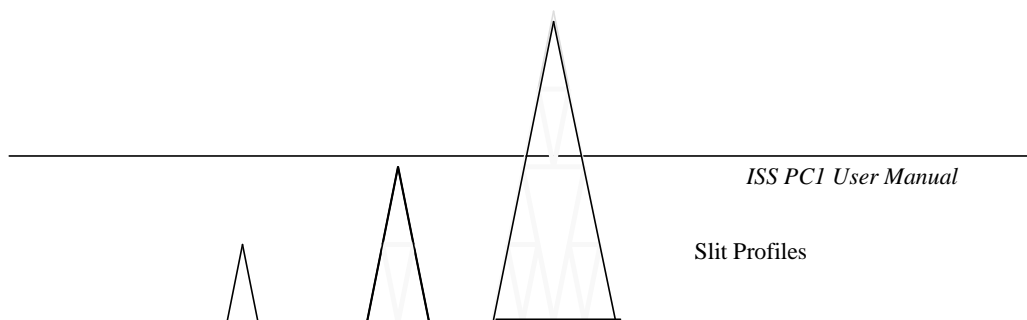
Always use a pair of slits with the same width to prevent a shoulder-shaped distortion of narrow spectral lines. The slit profile can be calculated from a convolution of entrance and exit slit intensity profiles and approximates a Gaussian shape, further approximated with a triangle. The wider the slit, the higher the intensity and the better the S/N.

#### Slit Profile and FWHM Bandpass

In selecting a suitable slit width one has to realize that with 2 equally wide slits installed the convoluted image profile is approximately a triangle (Slit Profile). The Full Width at Half Maximum (FWHM) describes the effective line profile width for an imaginary rectangular profile with the same area as the triangle.

Slits can be exchanged to alter excitation intensity and spectral resolution: The smaller the slit the less light passes but the better the spectral resolution.

Take this slit profile into account when you design an experiment. For a set of chosen excitation and emission wavelengths the slits used may create a partially overlapping excitation and emission, wrecking havoc with polarization measurements by weakly present scattered excitation light.



**Figure 4.1-12 Illumination Intensity at the Sample for Various Selected Slit Widths**

Select equal slits for the H10 monochromator i.e. 1 and 1 mm or 2 and 2 mm for the entrance and exit slits of the excitation monochromator. The wider the slit the lower the spectral resolution but the more light enters the sample and the emission monochromator.

**Standard Slits**

The monochromators are standard equipped with fixed 2 mm, 1 mm, and 0.5 mm slits. The standard monochromator has a dispersion 8 nm per mm.

**Additional Slits**

Narrower slits are available upon request. Narrower slits (mm) carry a smaller spectral bandpass (nm). The choice of slits to be used in a measurement depends on a number of experimental parameters. Start by running the lamp at 15 - 18 Amps and placing the 1 or 2 mm slits on the monochromators.

Monochromator	H10		DH10	
Slit Width (mm)	Bandwidth (FWHM) (nm)		Bandwidth (FWHM) (nm)	
4	32		16	
2	16		8	
1	8		4	
0.5	4		2	
0.25	2		1	
0.1	0.8		0.4	

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0.025	0.2		0.1	
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**Table 4.1-3 Available Monochromator Slits**

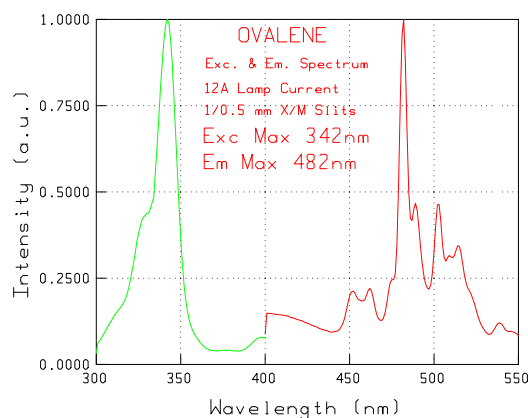
## 4.2 Intensity ↔ Wavelength Scans

### 4.2.1. Emission and Excitation Spectra

Spectra are acquired on the Monochromator emission channel. Start the ISSPC Spectra Software. Before you can start the data acquisition you should verify and enter into the computer the dial settings of the monochromators: wavelength verification of the monochromator dials with the aid of the <F8> screen. You should also specify where you want to store the data, <5> in the main spectra menu. Please refer to the Daily Operations section at the beginning of this manual.

An emission spectrum keeps the excitation wavelength fixed and scans the emission monochromator. The fixed excitation wavelength is often positioned at maximum absorption.

For an excitation spectrum one scans the excitation monochromator and keeps the emission monochromator at a fixed wavelength. The fixed emission wavelength is often chosen at the maximum of the emission spectrum intensity.



**Figure 4.2-1 Excitation and Emission Spectrum of Ovalene in PMMA matrix  
Selection of Optimum Excitation and Emission Wavelength**

From the measured absorption spectrum one can deduce the best excitation wavelength by choosing the wavelength corresponding with the maximum of absorption. Occasionally samples can not be diluted. The Optical Density (OD) at the peak absorption wavelength is larger than about 0.15 / cm. You may use a smaller dual optical path cuvette. You may also choose an alternative excitation wavelength near the peak absorption but with lower OD, i.e. smaller inner-filter effects. Take an excitation spectrum if the optimum excitation wavelength is unknown.

Acquire an emission spectrum (the excitation monochromator is kept at a fixed wavelength while the emission monochromator scans). Start always about 10 nm above the excitation wavelength to evade most of Rayleigh scattered excitation light (elastic scatter). Stop at a point where the fluorescence emission is weak (to longer wavelengths, to the red). If you see structure in the spectrum and you think that it can be further resolved, use 0.5 mm slits for the emission and excitation monochromators. When the signal is too weak and noisy, increase the intensity of the excitation light by increasing the current running through the lamp, 18A gives the best intensity stability. If the intensity is still not strong enough, replace the 0.5 mm slits of the excitation monochromator with 1 mm slits etc.

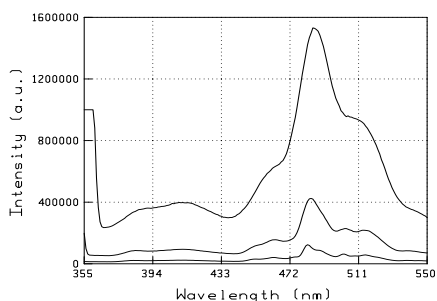
**Trade-Offs**

You will encounter situations, where spectral information content has to be balanced with Signal-to-Noise ratio / scan speed / stability of the sample:

Spectral Information  $\Leftrightarrow$  Optimum Intensity  
Resolution  $\Leftrightarrow$  Signal-to-Noise Ratio

Example:

Ovalene emission spectra for 0.5, 1 and 2 mm slits. Wider slits lead to less spectral information, but the more light is detected the faster the measurement proceeds and the better the S/N ratio.



**Figure 4.2-2** Ovalene Spectra for 0.5, 1 and 2 mm Emission Slits

**Adjusting the Excitation Intensity**

Sample illumination intensity can be adjusted in several ways. In the table the various possibilities are listed. Dependent on the experiment one or more methods can be applied.

Intensity Adjustment Method	Reproducibility	Means
Lamp current	Good	Current indicating dial
Excitation Monochromator Slits	Good	Marked slits
Absorption Type Filter	Good	Known transmission
Interference Filter	Good	Known transmission
Neutral Density Filter	Good	Calibrated transmission
Copper Screen(s)	Not very good	Mesh position
Excitation polarizer	Good	Always about 50% reduction (v)

Iris Diaphragm	Not very good	Lever position
Focusing lens position adjustment	Not very good	Adjustment knob

Table 4.2-1 Intensity Adjustment in Optical Spectroscopy

### Technical Spectra and Corrected Spectra

Spectra taken without implementation of any or all corrections are called technical spectra. When you look at changes from one sample to another this is often adequate. However, when you want to compare your data with spectra taken on other instruments you may want to correct for instrument effects: corrected spectra.

Spectra are automatically corrected for intensity fluctuations of the light source when R (ratio) detector mode is selected in the measurement set up screen: emission channel signal intensity is corrected with the reference detector constantly monitoring the light source intensity. See the Algorithm section for more information about the Ratio mode.

### Correction for Lamp Intensity Fluctuations

Light source intensity fluctuations are monitored via a quartz beamsplitter - Quantum Counter combination and the reference detector. The output of the emission detector is ratioed and re-normalized with the signal from the reference monitor photomultiplier detector (PMT). This ratio gives a value, which is independent of any changes in source intensity with time. Ratio mode acquisition has to be selected from the setup menu. Both reference and sample signals should have sufficient intensity to give a good Signal-to-Noise value.

When the reference signal is very small, the ratio will show the statistical fluctuations of the reference signal notwithstanding a very stable sample signal. In this way spectra will be corrected for distortions introduced by the lamp spectrum but not for the noisy response of the Reference PMT. Fluctuations in the photon signals are proportional to the square root of the observed number of counts/sec.

Example: A signal of 100 +/- 10 counts/sec has a 10% variation, a signal of 10,000 counts/sec a variation of 1%. The peak of a spectrum should therefore have a slightly higher noise level as compared to the background signal far away from the emission peak.

### Corrections for Reference PMT Wavelength Dependent Response

To obtain a fully corrected emission spectrum, the Reference PMT must have a response, which is directly proportional to the quantum intensity of the incident beam irradiating the sample. This is achieved using a quantum counter with a red filter and additional neutral density filters to attenuate the light reaching the reference detector.

### Quantum Counter and Quantum Counter Solutions

A compound whose quantum yield and emission maximum are independent of the excitation wavelength, temperature, aging of the dye has to be used in a quartz triangular cuvette. A highly concentrated solution of Rhodamine B in ethyl alcohol or in ethylene glycol (5-8 g/l) is a convenient quantum counter. This strongly absorbing solution provides a signal, proportional to the number of the incident photons. A red filter, e.g. a 2 mm thick 2 inch square RG-630, isolates the Rhodamine emission above 610 nm. Thin mesh neutral density filters may be required to reduce the light intensity. See the Reference section at the beginning of this manual for specific quantum counter literature. For experiments in the region from 600 to 900 nm and above, dyes like HITC (Hexa-methyl Indo Tri-carbo Cyanine) have to be used.

**Correction of Excitation Spectra**

This feature is automatically obtained by using a quantum counter in the Reference channel. After passing through the excitation monochromator, part of the incident light beam is diverted by a beam splitter to the Reference monitor channel.

**Correction of Emission Spectra**

These may be required for the wavelength dependent response of the detection system (collecting and focusing lenses, emission monochromator with its grating and slits, and also the emission channel Photomultiplier (PMT) detector). The wavelength dependent sensitivity of the instrument is measured for each instrument before shipping. Liquid standards like Quinine Sulphate Dihydrate and a spectral irradiance standard lamp (The Eppley Laboratory, Inc. or Oriel) can be used to determine the spectral response of the detection system. Calibration data are referenced to NIST, Gaithersburg, MD derived calibration data.

**Correction Files**

The correction files are provided for each instrument with the ISSPC Spectra Software. Correction of the emission spectra can be selected to be automatically performed at the moment of data acquisition. The user can decide whether or not to use the correction files at the time of spectrum acquisition. The software allows to correct stored spectra afterwards. Several types of monochromators with different gratings are in use. Each requires a different correction file.

Indeed, an inspection of the correction factors reveals that the ISS detection system does not introduce systematic errors larger than 5% in the emission maxima position when no correction factors are used.

**Measurement Mode: Ratio, Single or Double Mode (R, S, D)**

**R** (Ratio) mode data correct for lamp intensity fluctuations and also for detector spectral response when a quantum counter is used.

**S** (Single) channel data can be taken by setting the detector mode to S (Single). Only the sample detector signal is stored. The software allows you to swap channel signals on the screen. Therefore reference detector signals can also be recorded in the Single detector mode.

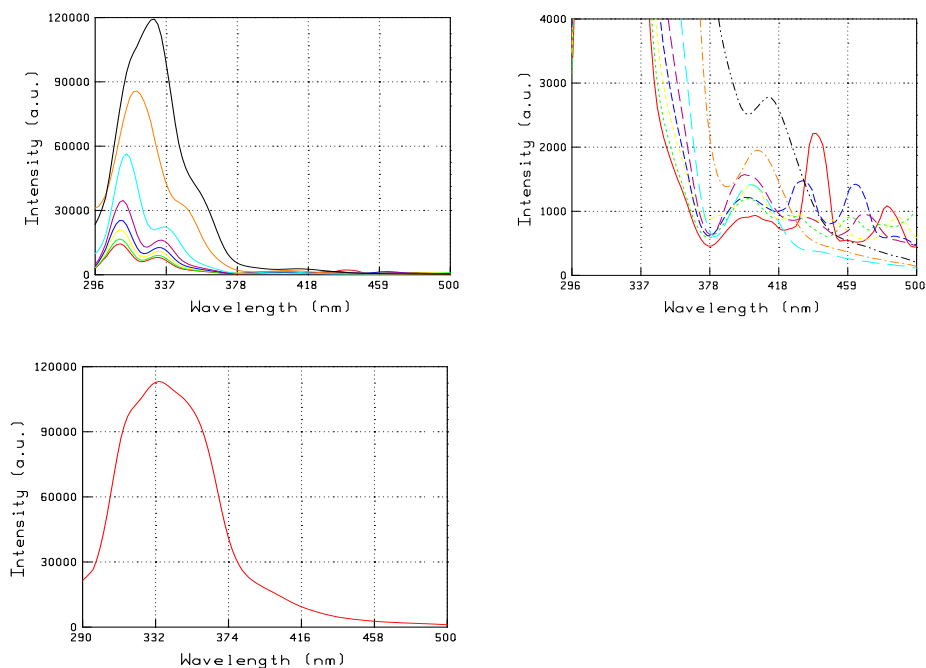
In the **D** (Double) detector mode you can collect and store data of two detectors without ratioing them during the measurement. Both detector signals are stored in a single file. Later you can decide whether and how to process the data.

**Background Subtraction**

Background emission subtraction is feasible when a second cuvette with just the buffer or other blank solution is inserted in the R(eference) cuvette position in the sample compartment. Set the Use Background subtraction switch in the experiment setup screen to Y. Use <ALT-A> to set the number of cuvettes to 2.

**4.2.2. Synchronous Luminescence Spectra**

Excitation and emission monochromators move at the same speed but one tracks the other by a given wavelength difference of 0.5, 1, 2, 3, 5, 10, 20 and 30 nm for example with 12A lamp current and 0.5mm excitation and emission slits, see figure. The emission monochromator is set a few nanometers ahead of the excitation monochromator. A signal will appear in the region where the excitation and emission spectra of the fluorophores overlap. This technique is used extensively for analytical purposes: when you have a mixture of three or four or more components as is usually the case for oil-industry samples. The compounds can be recognized from the properties of the overlapping region of their excitation and emission spectra.



**Figure 4.2-3** Upper Curves: Synchronous Spectra of Motor Oil. Lower Curve: Emission Spectrum of 10W-10 Motor Oil. Excitation Wavelength 280 nm and 0.5 mm Slits

#### 4.2.3. Excitation and Emission Matrices

Although one can perform these measurements one after the other in their proper sequence it is far easier to construct a Macro routine to automatically collect, process and store all spectra. See the Chapter detailing Macro programming and the example supplied. We recommend that you store the data in ISS (binary) format for saving disk space. Conversion to ASCII of all collected data files is also done with a macro routine.

The Macro programming section shows an example of a batch file that automatically converts ISS structured files into ASCII files. With an editor you may elect to clip the alpha-numeric header from the ASCII file. The numeric leftover can be ported to other advanced display and processing programs.

### 4.3 Intensity ⇔ Fixed, Single Wavelength

Questions answered by this type of measurement are for example: Is the enzyme still active after months of storage at low temperature? Is the protein not denatured after storage? Does this preparation give the same spectral peak intensity as a previous preparation? The answer to these questions is obtained by measuring the sample intensity at a single, fixed excitation or emission wavelength. The usual, user-selectable features of background subtraction and data collection to a given accuracy for a maximum amount of time are incorporated in the software with automatic printing / plotting. One of the toggle features is automatic closing of the excitation shutter to diminish sample bleaching between measurements followed by ASCII format data storage for these data collection modes:

#### 4.3.1. Single Point: Sample Conditions do Not Change with Time

Intensity measurement with automatic stop at a predetermined accuracy, Standard Deviation (STD). This measurement will go on as long as the preset accuracy has not been reached and the preset maximum allowed time has not expired. The left most column contains the instantaneous data. Right most data display the long-term average values. Differences in the values indicate changing sample conditions which would require slow-kinetics routines.

Background emission subtraction is feasible when a second cuvette with just the buffer or blank solution is inserted in the R(eference) cuvette position in the sample compartment. <F9> will change the measurement time (TB, time base on the top line of the screen). Integration time is a multiplier for the timebase. The next measurement starts when the difference between timebase x integration time and repeat time has elapsed. As said between the measurements the excitation shutter may be closed to minimize sample bleaching. Read also the Algorithm section.

### 4.4 Polarization ⇔ Wavelength Scans

#### 4.4.1. Introduction: Polarization Measurements

Slide the Glan-Thompson polarizer holders of both the excitation and emission channel into the optical path. Activate in the Advanced Options list the left or right emission polarizer. Place a polarization rotator (half wave plate, cellophane or similar) in front of the excitation shutter or polarizer, when you have a vertically polarized laser light source. This laser light source should be equipped with Brewster windows to create vertically polarized emission. Lasers usually emit vertically polarized light (unless the head is rotated 90 degrees) when Brewster-angle windows are present. Please use Glan-Taylor, air-spaced polarizers in the excitation optical path when laser power levels exceed several hundred mW/cm<sup>2</sup>.

A certain amount of horizontally polarized intensity is necessary to measure the polarization bias of the emission channel (g-factor).

#### Polarizer Side to Use

Before starting a measurement please use the <ALT-A> keys to check which emission polarizer will rotate, the left or right side one. Many instruments have a right emission monochromator. The left side is then usually a filter side. This emission filter side usually gives much more intensity than the emission monochromator side. Monochromators do not pass as much light as a bandpass, cut-on, cut-off or interference filter.

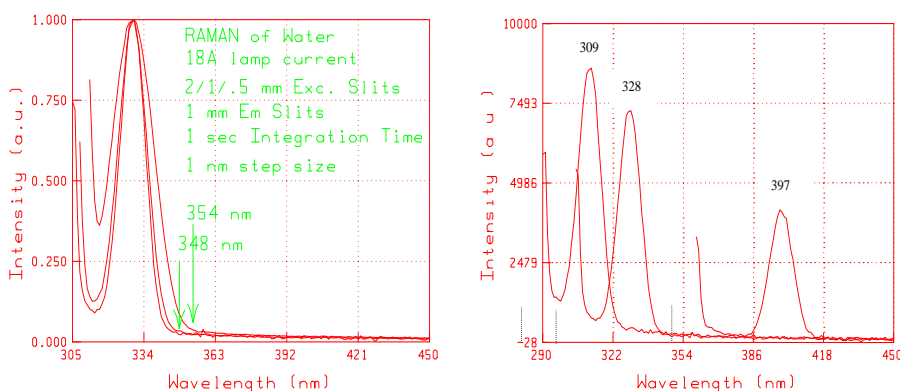
#### Elimination of Elastically Scattered (Rayleigh) Excitation Light

Select proper emission filters. Time and again scattered, vertically polarized, excitation light corrupts the VV intensity measurement. To test absorption- or interference-type emission filters use a cuvette filled with a glycogen scatter solution and check that the dark (emission shutter closed) detector intensity equals the shutter-open intensity. When no appropriate filter is available one may be able to change the chosen excitation or emission wavelength to a value, which does not cause problems. Be aware that also second order light passing through the excitation

monochromator may have to be eliminated. This second order excitation can be found at 2 x excitation wavelength and shows the characteristic triangular shape of the slit profile.

### Elimination of In-elastically Scattered (Raman) Light

One may select Raman notch filters or interference filters in emission to eliminate the Raman contribution to the spectral intensity. The strong Raman peak of water (a very common buffer medium) is always close to the excitation wavelength and is highest in the UV with 260, 280, and 295 nm excitation for DNA and protein experiments.



**Figure 7-71** Influence of Choice of Slit Widths and Excitation Wavelength on Raman Line Profile

You see that the choice of narrower excitation slits sharpens the Raman line profile considerably. When superimposed on a protein spectrum, a narrower Raman line is more distinct. For the widest excitation slits you can see that the Raman line superimposes with the red edge of the Rayleigh `scattered excitation line profile.

In the figure on the right you see that the distance of the Raman line to the excitation peak increases and the relative intensity decreases when the excitation wavelength is progressively chosen at 280, 295 and 350 nm.

Note: For weak samples the replacement of 10x10 mm emission Glan-Thompson polarizers by 14x14 mm ones will result in a gain of a factor 2 in transmitted intensity.

Note: The Raman band of water is found at approx.  $3400\text{ cm}^{-1}$  from the excitation wavelength expressed in wavenumbers.

### Integration Time

Select at least a 2 second integration time in the <ALT-A> screen. Take a value of 2 seconds. This will guarantee a satisfactory S/N.

### Signal Intensity and Saturation Effects for Photon-Counting Electronics

Signal levels for a selected lamp intensity and slit combination may be saturating one or more of the four measured polarization intensities, but typically  $I_{vv}$ . Use narrower slits, a reduced lamp intensity, a 10% or 1% neutral density filter in excitation or close partially the iris diaphragm behind the sample compartment to verify that the Polarization and anisotropy data keep the same value.

**Sample Temperature**

The rotation of molecules and the rate of chemical association / dissociation reactions etc. is very sensitive to the sample temperature. Please use a bath, firmly attached, short and well-insulated hoses and stirrers.

**4.4.2. Polarization (Anisotropy) Spectra**

A polarization spectrum requires the acquisition of four different excitation spectra at each wavelength. Each spectrum is obtained with the excitation and emission polarizers oriented differently and it is separately stored. Four spectra 1-4 are obtained with the excitation-emission polarizer orientations: HH, HV, VV, VH; H = Horizontal, V = Vertical

**Polarizer Principal Axis**

The polarizer principal axis is vertical when the set screw of the polarizer holder points upward.

**Grating (g) Factor for an Emission Channel**

g-factor: Correction for Emission Channel Polarization Bias

HH and HV determine the g-factor;  $g = I_{hv} / I_{hh}$ , for the emission channel. This factor is dependent on emission wavelength, slit width and detector characteristics in spectral regions where the instrument parameters are not wavelength independent.

**Automatic Calculation of Polarization Spectra**

A routine in the ISSPC Spectra Software, located under <3> Record Operations in the Main Spectra Menu, retrieves the files and calculates the polarization (or anisotropy) spectrum. Please enter the proper spectra identification numbers obeying the HH, HV, VV, VH sequence.

**4.5 Polarization ⇔ Single, Fixed Wavelength****4.5.1. Polarization (Anisotropy) Data Taken at a Fixed Wavelength**

The measurement of polarization at fixed wavelengths is straightforward once the proper filters, slits and temperature have been selected. Read the introduction of the previous paragraph about polarization spectra. The same considerations apply in obtaining the correct optical conditions. The software guides the user step-by-step through the operations. A couple of remarks are in order.

**L-Format and T-Format Optical Geometry**

Polarization measurements can be acquired using two different configurations: the L-format and the T-format. T-Format measurements will reduce the total data collection time by a factor of 2. Left and Right emission channel optical conditions should be as similar as possible for T-Format data collection. Conditions where the left emission side (filter) is 100x brighter as the right emission monochromator side should be avoided. The g-factor will be large, making the results maddeningly sensitive and inaccurate. You may opt to use "0" order and neutral density filters to equalize signal levels. The software has to be set to the specific configuration chosen for the measurements. Modify the <ALT-A> screen options where required.

**1. L-format Configuration**

One light detector is present on the reference channel and the other detector collects the fluorescence from one of the two emission channels (usually the one without monochromator). We suggest using the filter channel because transmission through a filter is usually higher than transmission through a monochromator.

In the L-format configuration, you need two polarizers, one for the excitation and one for the emission channel. g-factors are measured with the automated polarizers (or values for the g-factor are declared). Data are collected by rotating the polarizer in the emission channel to the (V) Vertical position and subsequently to the (H) Horizontal position. Data from the emission channel can be put into ratio with the light source monitoring reference channel.

Note: Once again, please select proper emission filters. Scattered, vertically polarized excitation light may otherwise corrupt the VV measurement.

## 2. T-format Configuration

When an instrument has only 2 detectors the photomultiplier tube that is usually placed in the reference channel is moved to the other emission channel.

For **T-format measurements in analog mode** follow the instructions on the screen to connect the 2 channels provided by the analog card to the PC1 instrument.

To prepare for a T-format measurement with three polarizer assemblies slide them in position, one for the excitation channel and one for each emission channel. The polarizers on the right and left emission channels are automatically set by the software to the (V) Vertical and (H) Horizontal positions respectively. In this configuration, the acquisition time of the measurement is cut in half.

### Single Point Polarization: Sample Characteristics do Not Change with Time

Intensity measurement with automatic stop at a predetermined accuracy, Standard Deviation (STD). This measurement will go on as long as the preset accuracy has not been reached. The left most column contains the instantaneous data. Right most data display the long term average values. Background emission subtraction is feasible when a second cuvette with just the buffer solution is inserted in the R(eference) cuvette position in the sample compartment. <F9> will change the measurement time (TB, time base on top of the screen). Integration time is a multiplier for the timebase. The next measurement starts when the difference between (timebase x integration time)  $\times 4$  and repeat time has elapsed. Between the measurements the excitation shutter may be closed to minimize sample bleaching.

### Polarization Measurement with Blank Subtraction

When studying highly scattering samples, the polarization measurement will more likely be affected by scattered light. Select proper filters. Background introduced by the buffer solution hosting the fluorophore may be more strongly present. The PC1 Spectrofluorometer allows you to determine the polarization corrected for the influence of the buffer or blank solution.

You need to prepare two cuvettes, one containing the buffer (blank) and the other containing the buffer with the fluorophore.

Your PC1 Spectrofluorometer is standard equipped with a **two-cuvette sample compartment**. Place the cuvette with the fluorophore in the sample slot, S, and the one with the blank in the reference slot, R. In the Parameter Setup, <Alt-A>, of the ISSPC Spectra Software you must set the software to the new configuration: 2 cuvettes in use. The measurement on the sample is acquired first: four data sets, each corresponding to a relative orientation of the excitation and emission polarizers, HH, HV, VV, VH, are stored in the computer. Next, the turret automatically rotates and the measurements on the blank solution are acquired. These four data sets are subtracted from the corresponding data collected on the sample. Finally, the polarization value is calculated from the data obtained.

## 4.6 Intensity $\leftrightarrow$ Time

### 4.6.1. Slow Kinetics Routines for Changing Sample Conditions

Bleaching of a Fluorescein dye, for example, can readily be followed by this routine. A repeat measurement will be carried out for the time period set by <F9> and the integration time multiplier. <F9> will change the measurement time (TB, time base on top of the screen). The next measurement starts when the difference between timebase X integration time and repeat time has elapsed. Total data collection time is entered. A stop criterion based on a preset standard

deviation value is meaningless here since the repeat time is likely in conflict with it. Between the repeated measurements the excitation shutter may be closed to minimize sample bleaching.

Example: DPH in model membrane systems. Background emission subtraction is feasible when a second cuvette with just the buffer or blank solution is inserted in the R(eference) cuvette position in the sample compartment

Note: The repeat time is counted from the moment that the just finished (previous) measurement started. If the repeat time is shorter than the integration time the program will immediately continue with the next measurement.

## 4.7 Polarization ⇔ Time

### Slow Kinetics Routines: Changing Sample Characteristics or Conditions

Binding of a dye to a protein or the unfolding and refolding of a protein, even the changes in observed polarization or anisotropy values caused by a release of the bound dye are monitored by this routine. For measuring slowly varying rotational effects the same considerations apply as for measurements monitoring the temporal behavior of the sample intensity. Naturally, do not forget to slide the polarizers in position. Check under the advanced options screen whether the correct L- or T-format geometry has been chosen and whether the correct polarizer side has been selected. The software allows to let you measure automatically the g-factor correcting the polarization bias, when you select N for entry of the g-factor value: i.e. measure it.

The time period is set by <F9> and the integration time multiplier. <F9> will change the measurement time (TB, time base on top of the screen). The next measurement starts when the difference between timebase X integration time and repeat time has elapsed. Total data collection time is entered. A stop criterion based on a preset standard deviation value is meaningless here since the repeat time is likely in conflict with it. Between the repeated measurements the excitation shutter may be closed to minimize sample bleaching. Example: DPH in model membrane systems. Background emission subtraction is feasible when a second cuvette with just the buffer solution is inserted in the R(eference) cuvette position in the sample compartment.

Note: The repeat time is counted from the moment that the just finished (previous) measurement started. If the repeat time is shorter than the integration time the program will immediately continue with the next measurement.

## 4.8 Intensity ⇔ Concentration

### 4.8.1. Slow Kinetics / Titration Measurements

With the PC1 Spectrofluorometer you can detect a decaying or increasing fluorescence intensity versus time. The time scale can be varied from two milliseconds up to several hundred hours. Intensity changes of a sample subjected to chemical modification can be followed.

The standard hardware configuration is used: a light detector in the reference channel and a detector on either one of the emission channels.

Note: A septum lid is available for repeated injection of aliquots. An automated titrator and software for use with the ISS PC1 Spectrofluorometer is available from ISS. See the next section for details.

Note: For day long, repeatedly interrupted measurements one is advised to use the light source at 18A for best stability. When observed effects are on the order of a few percent and in

the same range as the light source stability, one can reference the excitation intensity stability with a repeated measurement of an internal standard e.g. Raman line of water. A batch mode macro can be used for special experimental requirements, e.g. observation of the intensity of the second order intensity to monitor sample aggregation via an increase in scatter.

#### Titration Automation

A computer-controlled titrator is available from ISS for use with the ISS line of fluorescence instrumentation (PC1 Photon-Counting Spectrofluorometer and K2 Multi-frequency Phase and Modulation Fluorometer). In a typical series of titration measurements, the operator measures fluorescence parameters (excitation and emission spectra, polarization, and/or kinetics) after adding a specified quantity of a titrant (solution) to the sample contained in the cuvette. Delivery of the titrant is automatically performed by the titrator at time intervals specified by the operator. For instance, a series of polarization measurements can be performed on a sample whose concentration changes (titrant is added) every sixty seconds. See attached user manual.

#### 4.8.2. Fast Kinetics / Stopped-Flow Measurements

For Kinetic studies with a resolution of a few milliseconds, an electronic trigger has to start the mixing of your substances and the data acquisition as well. Additional hardware may be required for this mode of operation. This type of measurement can only be carried out with a special analog A2D card. See the chapter describing installation of accessories.

### 4.9 Intensity $\Leftrightarrow$ Concentration, pH, Pressure and Temperature

This type of measurements can be automated with several available instrument upgrades: automatic microprocessor controlled refrigerated temperature controller, pressure generator and titrator.

#### 4.9.1. Ratiometric Measurements

Data are collected at two different emission wavelengths for a chosen excitation wavelength. The opposite is also possible by entering M (dual excitation) instead of X (dual emission). This routine can be used for monitoring temperature and pH dependent effects on fluorescent probes sensitive to for example calcium concentration. In order to get the correct numerical values one has to determine the relative system sensitivity at both chosen wavelengths. One can look these values up in the correction files or measure them for a given wavelength and slit width for example by comparing the measured and absolute data for Quinine Sulphate Dihydrate (QSD).

#### 4.9.2. Generalized Polarization

Dipolar relaxation of molecules in a polar liquid or liquid crystal environment leads to mixtures of unrelaxed (blue) and relaxed (red-shifted) emission spectra. Examples: membrane probes Laurdan, Prodan. The fractional contribution of each component in different environments (phases) can be estimated by measuring the steady-state intensity at the blue and red edges of the spectral peak.

Generalized polarization, is very sensitive to phase transitions. It does not need an ad hoc calibration curve and the effect is additive. In order to get the correct numerical values one has to determine the relative system sensitivity at both chosen wavelengths. One can look the values up in the correction files, call the numerical values up or measure them for a given wavelength and slit width for example by comparing the measured and absolute data for Quinine Sulphate Dihydrate.

### 4.10 Polarization $\Leftrightarrow$ Concentration, pH, Pressure and Temperature

In above paragraphs the general considerations and precautions have already been outlined. These types of measurements can be automated with several available instrument upgrades: automatic microprocessor

controlled refrigerated temperature controller, automatic pressure generator and titrator. High pressure studies have to be corrected for the pressure sensitive optical window birefringence, which also shows hysteresis effects.

Notes:

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