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June, 1999
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CHAPTER I

Introduction

The FL-3D is a new generation spectrofluorometer which employs design for extremely fast data collection of the entire excitation/emission matrix for a fluorescent sample. The system may be used to find excitation and emission maxima for unknown samples and perform various kinetics measurements based on temperature, reactions and other parameters. Datamax-3D Spectroscopy Software, which is based on Grams/32c® from Galactic Industries, is used for all instrument control, spectral collection, data manipulation and presentation.

Manual Conventions

The typographical conventions used throughout this manual are presented in the following table.

<table>
<thead>
<tr>
<th>Item</th>
<th>Text</th>
<th>Example</th>
</tr>
</thead>
<tbody>
<tr>
<td>Software menu items</td>
<td>Define Experiment</td>
<td>Select Define Experiment</td>
</tr>
<tr>
<td>Sequence of menu items</td>
<td>Define Experiment/ Excitation Scan</td>
<td>Execute the menu sequence Define Experiment/Excitation Scan</td>
</tr>
<tr>
<td>Hardware controls</td>
<td>Start</td>
<td>Press the START button</td>
</tr>
<tr>
<td>Filenames</td>
<td>Mcorrect</td>
<td>Save the file as MCORRECT</td>
</tr>
<tr>
<td>Field Name</td>
<td>Scan Units</td>
<td>Toggle Scan Units to Nanometers</td>
</tr>
<tr>
<td>Keyboard Key</td>
<td>Enter</td>
<td>Press ENTER</td>
</tr>
</tbody>
</table>

Table I-1: Text Conventions
Additional symbols and conventions are used to draw your attention to special conditions. They are presented below.

<table>
<thead>
<tr>
<th>Symbol/Annotation</th>
<th>Meaning</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="lightning_bolt.png" alt="Lightning Bolt" /></td>
<td>Hazardous condition(s) exists. Further information regarding the hazards and precautions follow the symbol within this document.</td>
</tr>
<tr>
<td><strong>Warning</strong></td>
<td>Warnings appear wherever there is a danger that improper execution of a procedure may damage the equipment. ISA is not responsible for damage arising out of improper handling of equipment.</td>
</tr>
<tr>
<td><strong>Important</strong></td>
<td>This annotation is used to draw your attention to general information that should be considered. It appears throughout this document.</td>
</tr>
</tbody>
</table>

*Table I-2: Special Symbols*

This manual is divided into logical sections of information, and it includes step-by-step directions for performing certain operations. A brief description of each chapter follows:
Chapter Overview

Chapter 1

Introduction
Gives a broad overview of the system operation, describes how this manual is organized, and outlines where information can be found.

Chapter 2

Installation Instructions
This chapter describes how to install your instrument. System setup, cable connections and software installation are discussed here.

Chapter 3

System Description
Discusses how the system works, describes each component and introduces Datamax-3D software.

Chapter 4

Getting Started
Explains how to initialize your system. Step-by-step instructions tell you how to turn on the system and check system operation.

Chapter 5

Acquiring Data
This chapter presents the various features of the Datamax–3D software for the FL-3D instrument. The various data collection modes available on your system are explained in detail.

Chapter 6

Optimizing Your Results
This section concentrates on getting the best results possible out of your instrument. Optimizing system settings and scan parameters are discussed. Sample collection methods and choices for scans are explored for the user.

Chapter 7

A Tutorial Acquisition
Go through a basic experiment using the FL-3D to become more familiar with the instrument.

Chapter 8

Accessories
In this chapter you will find a description of each optional accessory available for the FL-3D.

Chapters 9

Maintenance
Routine maintenance procedures are discussed here to keep your FL-3D running properly.

Chapter 10

Troubleshooting
Potential sources for problems encountered in instrument installation or operation are reviewed with the symptoms and possible solutions.

Chapter 11

Technical Specifications
Details and specifications for FL-3D components

**Appendix A**

*Bibliography*
List of useful references dealing with fluorescence spectroscopy.

**Appendix B**

*Glossary*
List of useful terms related to fluorescence spectroscopy.

**Appendix C**

*GPIB Boards and IEEE-488 Installation Notes*
Instructions for GPIB Card (IEEE-488) installation and setup.

**Appendix D**

*Spectral Calibration Procedure for the FL-3D*
Procedures for calibrating the Spex 3D system across the excitation/emission matrix.

**Appendix E**

*Datamax-3D Initialization Files*
Printouts and explanations for important configuration files in Datamax-3D.
For Technical Assistance or More Information

The GRAMS/32® software manual describes all data handling and post-processing functions available in the software.

Technical support is available for both hardware and software troubleshooting. Prior to contacting the service department, however, complete the following steps.

- **REBOOT.** If this is the first time the problem has occurred, try repowering your system. Turn off the system and accessories, then, after a cool-down period, turn the system back on and see if the failure persists. Many apparent software problems are actually conflicts with other software or peripherals currently active on your system.

- **CONNECTIONS.** Make sure all accessories are attached and configured properly. Verify that line cords provide the proper input AC voltage, and that all appropriate accessories are powered ON.

- **TROUBLESHOOTING.** Review Chapter X, *Troubleshooting*, to see if your problem is discussed in the text.

- **VISIT OUR WEB SITE** at [http://www.isainc.com/fluor](http://www.isainc.com/fluor) to see if your questions are addressed in the **Systems** or **FAQs** (Frequently Asked Question) sections of the site.

- **DUPLICATE.** Make an attempt to duplicate the problem and write down the steps required to do so. The service engineers will make an attempt to do the same with a test system. Depending on the nature of the problem a service visit may not be required.

- **NOTE THE ERROR.** If an error dialog box pops up in DataMax-3D, write down the exact error displayed.

- **SOFTWARE VERSION.** Access DataMax-3D and from the Help/About menu at locate and note the version of the Grams/32 software.

- **SYSTEM CONFIGURATION.** Make a note of the instrument’s serial number and instrument configuration, including all accessories.

If you require further assistance after reading the manuals, and are in the United States, please call us at (732) 494-8660, fax at (732) 549-5157, or visit the web site and drop an e-mail to service. Customers outside the United States should contact their local distributor.

In the communication you should relay the information you noted from the steps outlined above. Without this information, it may be difficult for technical support to provide you with prompt assistance.
Keeping this document and the software manuals near the system and referring to them often will ensure that all needed information is readily available. A complete familiarization with the hardware and the software is necessary to avoid unneeded service calls. The system and software are designed to perform optimally with minimum care and maintenance. If, however, a problem is detected, refer to the Troubleshooting and System Maintenance sections of this manual as well as the Grams/32 software manuals prior to contacting the Fluorescence Service Department. Often, the manuals will reveal the cause of the problem and a solution; thereby eliminating the need for service.

The remainder of this manual details the operation and maintenance of your FL-3D spectrofluorometer.
CHAPTER II

Installation Instructions

The FL-3D spectrofluorometer system is delivered in a single packing carton. The carton is designed to provide maximum protection during shipping, while allowing easy access to the system to facilitate unpacking. If a host computer (PC) has been ordered as a part of the system, the PC will be delivered in a few clearly labeled boxes. All accessories, cables, software and manuals ordered with the system are included with the delivery.

Examine the shipping boxes carefully. Any evidence of damage should be noted on the delivery receipt and signed by representatives of the receiving and carrier companies. While Instruments S.A., Inc., is not responsible for damage occurring during transit, the company will make every effort to aid and advise.

Selecting a Location

Before unpacking the FL-3D, select its permanent location. For proper operation, the location should include the following.

1. A sturdy table capable of supporting a mass of at least 36 kg (80 lbs.) and possessing proper dimensions for the FL-3D system. (at least 21"X 24"X12.5" (lXwXh) for the spectrofluorometer, and 10"X12"X6" for the CCD-3000 controller). The system may be set up with the CCD-3000 controller located on top spectrofluorometer unit.

   **Important:**
   
   Don't forget to consider space and weight factors for your host computer and for your desired measurement area when choosing a table and suitable location.

2. Ambient temperature ranging from 15°C to 30°C (60°F to 85°F) with a maximum fluctuation of ±2°C.

3. An ambient relative humidity of less than 75%; excessive humidity can degrade the optics.

4. Low dust levels.
The system has an exhaust fan intake on the right hand side of the instrument with an exhaust in the back of the lamp housing. The instrument should be located with enough space around these fans to allow for proper ventilation.

Although proper physical parameters and environment are essential to the operation of the spectrofluorometer, the system’s electrical requirements must also be taken into consideration.

The FL-3D requires a line voltage of 110V/60Hz or 220V/50Hz. The line voltage must be maintained within ±5%.

Instruments S.A., Inc., is not liable for damage due to line surges and voltage fluctuations. A surge protector is strongly recommended for minor power fluctuations. For more severe voltage fluctuations, a generator or an uninterruptible power supply (UPS) is suggested. Improper line voltages can severely damage the equipment.

Your instrument is equipped with a three-conductor power cord that is connected to the system frame (earth) ground. This ground provides a return path for fault current due to equipment malfunction or external faults. For all instruments, ground continuity is required for safe operation. Any discontinuity in the ground line can make the instrument unsafe for use. Do not operate this system from an ungrounded source.

The following list contains all of the components, which must be connected to an AC power source.

- Computer (CPU)
- Monitor
- Printer (optional)
- FL-3D spectrofluorometer
- CCD-3000 controller

Make sure enough outlets are available in the selected installation area prior to setting up the system.

**Unpacking and Installing**

Once a location has been decided upon, unpack and assemble the equipment. To avoid excessive moving and handling, the equipment should be unpacked as close as possible to the selected location.
Warning!

The FL-3D spectrofluorometer system is a delicate analytical instrument. Mishandling may seriously damage its components. Closely follow directions for cabling the system as the CCD is extremely sensitive to static electricity when ungrounded.

It is important to note that many public carriers will not recognize a claim for concealed damage if it is reported later than 15 days after delivery. In case of a claim, inspection by an agent of the carrier is required. For this reason, the original packing material should be retained as evidence of alleged mishandling or abuse. While Instruments S.A., Inc., assumes no responsibility for damage occurring during transit, the company will make every effort to aid and advise.

Follow the instructions presented below to unpack and assemble the system.

**FL-3D Spectrofluorometer**

The spectrofluorometer is contained in a single packing carton. Upon opening the carton, you will find:

<table>
<thead>
<tr>
<th>Qty.</th>
<th>Item</th>
<th>Part Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>SPEX brand 3D spectrofluorometer</td>
<td>FL-3D</td>
</tr>
<tr>
<td>1</td>
<td>CCD-3000 Controller</td>
<td>CCD-3000</td>
</tr>
<tr>
<td>1</td>
<td>CCD TE-Control Cable</td>
<td>37661</td>
</tr>
<tr>
<td>1</td>
<td>CCD Control Cable</td>
<td>35872</td>
</tr>
<tr>
<td>2</td>
<td>Power cord (115V)</td>
<td>98015</td>
</tr>
<tr>
<td></td>
<td>(220V)</td>
<td>98020</td>
</tr>
<tr>
<td>1</td>
<td>Datamax Software Package for FL-3D</td>
<td>Datamax-3D</td>
</tr>
<tr>
<td>1</td>
<td>FL-3D Users Manual</td>
<td>81037</td>
</tr>
<tr>
<td>1</td>
<td>IEEE-488 Card &amp; Cable (if ordered)</td>
<td>973008</td>
</tr>
<tr>
<td>2</td>
<td>Pack of Fixed Slits (.05, 0.1, 0.25mm)</td>
<td>351300</td>
</tr>
</tbody>
</table>

*Table II-1: Items supplied with FL-3D systems*

Proceed with the following unpacking instructions:

1. Carefully open the FL-3D shipping carton.
2. Remove the foam injected top piece and any other shipping restraints in the carton.
3. With a co-worker carefully lift the instrument from the carton and rest it on the lab bench on which you will be installing the system.
4. Place the instrument in its lab location.

5. Level the spectrofluorometer.

6. Inspect for previously hidden damage and notify the carrier and Instruments S.A., Inc. if any is found.

7. Check the packing list to verify that all components and accessories are present.

8. Remove the plastic tie wrap shipping restraint from the lamp compartment. The tie wrap is used to secure the lamp in the spring loaded mount during shipping.

   a. Unscrew the four screws securing the lid on the sample cover.

   ![Figure II-1: Removing the Lamp Housing Cover](image)

   b. While holding one end of the tie-wrap in one hand, use a pair of cutters to cut off the tie-wrap.

   ![Figure II-2: Removing the Tie-Wrap from the Lamp Housing](image)

   c. Replace the lamp compartment lid, and secure with the four screws.
Warning!

The CCD head, the Detector Interface Unit, the IEEE-488 board and the computer are very sensitive to electrostatic discharge (ESD). ESD precautions should be followed. The computer must be turned off, but its power cord should be connected to a grounded outlet to take advantage of the outlet ground.

Always turn the power off to all components before connecting or disconnecting any cables.

Before inserting a connector, touch the connector shell to the component case to discharge any accumulated static charge.

9. With the power switch in the OFF (0) position, plug one end of the power cord into the proper receptacle on the rear (facing the unit) of the spectrofluorometer.

CONNECTORS:

TOP: CCD Signal/Control
(25 pin connector on P/N 35872)

MIDDLE: MCD-Shutter from CCD-3000
(9 Pin Connector on P/N 35872)

BOTTOM: TE Control
(9 Pin Connector, P/N 37661)

RIGHT: Line Cord

Figure II-3: FL-3D Cable Connections

3:FL-3D

Figure II-4: CCD-3000 Cable Connections
9. With the power switch in the OFF (0) position, plug the female end of the power cord into the proper receptacle on the rear of the CCD-3000 controller.

10. Connect one end of the IEEE-488 cable to the IEEE-488 connector on the CCD-3000 controller.

11. Remove the grounding connector from the 25-pin CCD detector connector on the back of the FL-3D unit. Connect the 35872 cable's 25-pin connector to the CCD, first touching the cable connector shield to the metal case of the FL-3D to drain any static charge.

**NOTE:** To prevent Electrostatic Discharge (ESD) damage, insert the grounding plug into the CCD detector connector on the FL-3D whenever the CCD head is disconnected.

12. Connect the 9-pin shutter that branches off the 35872 cable to the shutter connector on the FL-3D (the first 9 pin connector below the 25 pin connector). The 9 pin shutter connection on the FL-3D only has two active wires. The 7 unused pins on this connector are sealed.

13. Connect the 37661 TE control cable between the TE cooler supply connector on the CCD-3000 and the TE connector (lower 9 pin connector) on the FL-3D.

The FL-3D spectrofluorometer is now connected. The remaining steps involve configuration the IEEE-488 communication from the host computer to the FL-3D and installing the Datamax software package.

**Computer**

The information gathered by the FL-3D system is both displayed and controlled through the host PC with Datamax software for the 3D. The host PC may be purchased from Instruments S.A., Inc., or another supplier. In any event, however, the computer and its accessories will be delivered with assembly instructions from the PC manufacturer.

Set up your host PC reasonably close to the FL-3D system. The limitation will be the length of the IEEE-488 communication cable. The recommended location for the PC is just to the side of the spectrofluorometer but is by no means the only possibly orientation.

Follow the instructions, which came with the host PC to set up the computer system including the CPU, monitor and related peripherals (keyboard, mouse, speakers, printers, etc.).
**GPIB Communication**

If your host computer was purchased from Instruments S.A. then the GPIB interface card is shipped to your lab installed and pre-configured. However, if you have provided your own host computer than you will have to properly install and configure the GPIB card in your host PC. Follow the instructions which came with the GPIB card for installing the card in your PC and loading the appropriate drivers. Also, refer to Appendix C; *GPIB Installation Notes*, for important reference information on installing these cards for IEEE-488 communication.

**System Interface**

With all the equipment unpacked and the GPIB card installed, the only steps remaining are to interface the computer to the FL-3D spectrofluorometer, set up any automated accessories and install the software.

14. Attached the free end of the IEEE-488 cable, which is attached to CCD-3000 controller to the GPIB board installed in the host computer.

15. Install any accessories that arrived with the system using the instructions that accompanied the accessory. (Refer to Chapter VIII for a detailed list of accessories.)

The spectrofluorometer is now completely assembled and ready for software installation.
Software

The spectrofluorometer system is controlled by Datamax-3D software operating within the Windows 95™ environment. If you have purchased the computer and software from ISA, the software installation has already been performed. If you purchased the computer from a different source, you will have to perform the installation.

Before the Datamax-3D spectroscopy software can be installed, however, Windows 95 or 98™ must already be installed and operating properly. Refer to your Windows™ manual that came with the computer for installation instructions.

The software is supplied on several disks comprised of Grams/32 software and the Datamax-3D Program and Configuration Disks. The disks within each set are sequentially numbered and during the installation procedure, you will be prompted to insert the appropriate disk into the floppy drive.

To install the Datamax-3D spectroscopy software,

1. Turn on the computer, and go to Control_Panel/Add/Remove_Programs

2. Insert Disk 1 of the Grams/32c® software package into the floppy drive (either A: or B:, depending on the configuration of the computer).

3. Click the Install button. Windows™ should find the SETUP.EXE program on Disk 1. If Windows can’t find SETUP.EXE, use the Browse button to point to the proper drive and directory on the floppy.

4. Begin the Installation and follow the instructions given.

5. Select the Complete Installation.

6. You will be asked to specify the directory name where the Grams/32 software will be installed. The default directory of GRAMS/N is recommended.

7. Follow the prompts on the screen to enter all of the disks for the Grams/32 installation.

8. When the Grams/32 installation is complete, go back to Control_Panel/Add/Remove_Programs. Insert the first disk of the Datamax-3D Program and Configuration Disk Set into the floppy drive.

9. Click the Install button. Windows™ should find the SETUP.EXE program. If Windows can’t find SETUP.EXE, use the Browse button to point to the proper drive and directory on the floppy.
10. This installation procedure for the Datamax-3D software will prompt you through the required disks to be installed. When asked for the directory for the software to be installed, enter the drive and path for the directory where the Grams/32 software was installed (GRAMSN by default).

The installation and setup of the FL-3D, host computer and software should take less than an hour. When complete, you are ready to begin using your new 3D spectrofluorometer! If problems are encountered during the installation, or subsequent to installation, refer to the Troubleshooting and System Maintenance sections of this manual for guidance.
CHAPTER III

System Description

This section describes the basic operating principles of the Spex 3D system, its primary components, and the DataMax-3D spectroscopy software that controls data acquisition.

Basic Theory of Operation

The FL-3D spectrofluorometer uses a novel design and state of the art components to provide you with unparalleled spectral collection capabilities. Excitation light of 250-550nm from a Xenon source is dispersed vertically and focused on less than 1cm of a sample cell. The resulting sample luminescence is collected by the emission spectrograph, dispersed horizontally and focused on a multichannel CCD detector.

The data collected on the CCD is transferred to the controlling software on the host computer using a separate controller and IEEE 488 interface. Data collection throughput has been optimized to allow for a full 300nmX500nm excitation/emission matrix (EEM) acquisition in less than one (1) second.

Once transferred to the host computer, Datamax-3D software can display and manipulate the full three-dimensional matrix of data (excitation $\lambda$, emission $\lambda$, and intensity value) using the power of Grams/32® software. Datamax-3D can also extract individual excitation, emission, or synchronous spectra as desired for individual spectral processing.

System Components

The optical system consists of the following main components: light source, excitation spectrograph, sample compartment, emission spectrograph, CCD detector, CCD controller and host computer. A discussion of each of these components follows.
**Illuminator**

The source is a 75 Watt short-arc ozone-free Xenon lamp with continuous spectral output from 250nm to above 1000nm. The lamp is mounted vertically with the anode on top and possesses an average life of 400 hours. The mounting is spring locked to allow for thermal expansion of the lamp during use and also for ease of replacement. Instructions on lamp replacement may be found in Chapter IX: Maintenance.

Xenon lamps are used because of their bright luminance, arc stability and wide spectral output through the ultra-violet to the near-infrared. This lamp is ozone free and only requires heat exhaust from the instrument. All short-arc lamps are filled to several atmospheres at room temperature, and have greater pressure during operation so great care should be used when maintaining or replacing these lamps. Follow the instructions in Chapter IX and those enclosed with the lamp when performing routine maintenance.

The lamp power supply is a 75 – 300 Watt power supply and is located next to the lamp inside the FL-3D. The supply automatically arcs the lamp when the FL-3D system is powered on. The metal instrument cover acts to suppress the EMF spike that is common with all short-arc lamp supplies.

**Excitation Spectrograph**

The excitation spectrograph is mounted to the left side wall of the instrument. It acts to disperse the excitation light, from 250-550nm, evenly upon 8mm of height at the sample cell position. The optics are as follows.

The excitation from the lamp is collected with an ellipsoidal mirror and directed to flat mirror and then upon the concave holographic excitation grating. The grating focuses the diffracted light upon the sample cell position.

**Gratings**

A concave holographic reflection grating (133 grooves/mm) is used for the core optic of the excitation spectrograph. The grating is blazed to have response from 300-800nm. Concave gratings are used improve throughput and imaging by allowing for aberration correction and focussing to be performed by the grating alone without requiring other optical elements. Holographic gratings are chosen to reduce stray light 'ghosts' seen in classically ruled gratings.

**Slits**

The Spex 3D comes with three fixed slit widths (0.05, 0.1, 0.25mm). Each slit is labeled for excitation (X) or emission (M). The main consideration in choosing slits is the balance of intensity observed versus spectral resolution. The wider the slits, the more
light intensity that will be incident on the sample and detectors, but at the price of lower spectral resolution. The narrower the slits, the better the resolution at the expense of signal intensity. Ideally, slits should be set for intensity to the high end of the linear region of the CCD detector, but with resolution sharp enough to discern the spectral features that are being investigated.

**Emission Spectrograph**

The emission spectrograph collects the fluorescence emission from the sample using a spherical mirror then directs it to a toroidal mirror for aberration correction. From the toroidal, the emission beam is directed off a flat mirror to the emission concave holographic grating (133gr/mm, response from 300-800nm). The dispersed emission matrix is focused across the CCD detector head.

The optics used have a large enough size to correctly image the entire (250-550nm Excitation, 250-750nm Emission) EEM across the CCD.

**Multichannel (CCD) Detectors**

A 512X512 pixel UV enhanced charge coupled device (CCD) detector is used. CCD detectors are essentially large area silicon photodiodes constructed such that the area is divided into a two dimensional matrix of pixels.

A high speed emission (MCD) shutter for the CCD is located behind the emission slit mount on the FL-3D. The use of this shutter determines the *exposure time* or *integration time* for the CCD acquisition. The shutter is closed at all times except during data acquisition. This protects the CCD detector from stray light when working with samples.

When illuminated by opening the MCD shutter, each pixel integrates the charge arising from the photoelectric effect. The charges of adjacent pixels are kept separated by a grid of electrodes that confine the charges by electrostatic force.

At the end of the signal integration time the shutter is closed. Then the electrode grid voltages are manipulated by control signals from the Detector Interface Unit. This will sequentially shuttle the pixel charges row by row or column by column to the edge of the chip into a read out register. Based on the controlling software’s settings, the Detector Interface Unit can cause the readout to be formatted as either individual pixel datapoints or as areas of several pixels binned into 'superpixels'. The signal from the CCD is processed, amplified and converted to digital datapoints by electronics in the Detector Interface Unit.

The data is passed from the Detector Interface Unit to the CCD controller. This allows the software running in the host PC to access it rapidly for further processing and display.
The readout rate of a slow scan scientific grade CCD is about 20 KHz. The CCD’s used in television cameras, where S/N is less critical, scan at about 60 Mhz.

**Thermoelectrically Cooled CCD Heads**

An air heat exchanger is provided for the thermoelectrically cooled CCD detector head on the Spex 3D. This head employs two-stage Peltier effect cooling devices inside a purged chamber. These TE cooled heads can run continuously at their set operating temperature without requiring liquid nitrogen. The TE heads use forced air ventilation to dissipate heat. If the heat sinks on the CCD should ever overheat (above 60°C), a sensor will signal the Peltier cooler power supply to shut down. In this way, damage to the head is prevented.

![Figure III-1: MTECCD-512X512-9: Air Cooled UV2 Coated, Backthinned CCD Head](image)
The CCD used (Part # MTECCD-512X512-9) is a backthinned UV enhanced square 512X512 element CCD. It has been chosen for its element size, response from UV through near infrared, and sensitivity. The spectral response for this chip is shown below.

![Spectral Response of the MTECCD-512X512-9 CCD](image)

**Figure III-2: Spectral Response of the MTECCD-512X512-9**

### CCD-3000 Controller

The CCD-3000 controller acts as a high speed link between the host computer with Datamax-3D software and the CCD detector. It controls the CCD head based on commands from Datamax-3D on the host computer. The unit supplies power, clocking signals, and biases to the CCD sensor array. The controller unit also amplifies and digitizes the signal as it is collected from the CCD.

The controller is designed to transfer data via IEEE-488 communication. A National Instruments GPIB IEEE-488 card should be received with the instrument or provided by the user. Appendix C: *GPIB Installation Notes* contains a list of compatible cards as well as installation and configuration information.

The power switch, IEEE 488 cable connections, and all CCD connections are located on the back of the CCD-3000 controller.
**Host Computer System and Datamax Software**

We recommend at least a Pentium (586) 90 MHz microprocessor with 16Mb RAM on the motherboard should be used for the host computer. The hard drive should possess at least 50Mb free space for the Datamax-3D programs (more is required for data storage). Also, a mouse, keyboard, SVGA monitor and proper video card should be part of the host PC setup. A printer is a useful option, or you may decide to install a network card to print via an ethernet link to a network printer.

Datamax-3D Spectroscopy Software is used for all interaction with the spectrofluorometer. This software provides the user with complete control for setting up, running, manipulating, analyzing and outputting their fluorescence analysis. It is based on GRAMS/32 software from Galactic Industries. This interface allows for rapid data collection as well as power data processing tools and import/export capabilities.

Chapter V: *Acquiring Data* explores the software features available for fluorescence acquisitions on the Spex 3D. You may also consult the GRAMS/32 manual for information on data processing, manipulation and display.

The design and optical layout of the Spex 3D provides the user with a highly sophisticated and versatile research grade spectrofluorometer which may be used for rapid spectral collection. With Datamax-3D software control, complicated and time-consuming fluorescence scans and assays may the performed at the touch of a button for a variety of applications. Following data collection, manipulation and presentation of complex three dimensional data can be done readily.

The development of the Spex 3D has resulted in a rugged yet highly sophisticated spectrofluorometer which is easy to use and maintain.

After becoming familiar with the design, operation, and optical configuration of the Spex 3D, you should now feel a certain level of comfort with the system. The following chapter will now help you 'get started' with taking fluorescence measurements using the instrument.
CHAPTER IV

Getting Started

The Spex 3D spectrofluorometer system consists of the FL-3D spectrofluorometer unit, the CCD-3000 controller and the host computer with Datamax-3D software for the FL-3D. This section explains how to power up your system and check system performance. In this process you will learn how to define and execute an acquisition as well as some of the different software features.

Powering Up Your System

The recommended power-up sequence for the Spex 3D is as follows. The FL-3D unit should be the first component turned on using the rocker switch on the back of the instrument. This switch powers the lamp power supply, cooling fans and high speed shutter. The Xenon lamp will be ignited immediately after powering on the FL-3D.

WARNING

The Xenon lamp can emit an EMF spike that can be harmful to the CCD-3000 controller as well as PC's located near the instrument. Always ignite the lamp prior to turning on the CCD-3000 controller or any PC's located next to the instrument or on the same electrical circuit. To help reduce this spike the lamp is enclosed in a Faraday cage.

Once the lamp is ignited, turn on the CCD-3000 controller by switching the rocker switch to the ON (1) position. The power indicator LED on the front of the CCD-3000 will be illuminated when on.

Allow about five minutes for the CCD to cool down to below 250K (-23°C) before running the software.

Start up the host computer and any peripheral devices. Double click on the GRAMS/32 icon (or Datamax-3D icon) or select the program from the Start menu to start the software.
First FL-3D unit Press the power switch on the rear of the FL-3D to the ON (1) position.

Second CCD-3000 controller Press the power switch on the rear of the CCD-3000 controller to the ON (1) position. Run for five (5) minutes to cool down the CCD detector before starting the software.

Third Peripherals Computer and Software Turn on all peripheral devices such as printers and plotters. Boot up the computer and access Windows™ Program Manager. From the Datamax folder (or the folder to which you installed Datamax), run GRAMS/32.

Table IV-1: Power-Up Sequence for the FL-3D Spectrofluorometer

When Datamax-3D software starts, the GRAMS/32 software application platform becomes active. To acquire data, open the Collect menu and select 3D_CCD.

Datamax-3D will now connect to the CCD-3000 controller via the IEEE-488 link and run the initialization procedure for the CCD head. This should take under one (1) minute. When complete, the 3D acquisition menu will be active.

Click on Options and verify that the CCD temperature is below 250K before running any experiments. If the CCD temperature is above this temperature you will observe higher background detector counts during your acquisitions. Wait for the temperature to drop below 250K, then exit and restart the software.

**IMPORTANT**

If there are any difficulties in starting the software or initializing the Spex-3D please refer to Chapter X: Troubleshooting for suggestions.

This chapter explained the startup procedure for the Spex 3D spectrofluorometer. The following chapter will discuss data acquisition and the options available to collect spectra with your Spex 3D spectrofluorometer.
CHAPTER V

Acquiring Data

This section describes data collection on the Spex 3D using the Datamax software. Setting up acquisitions, adjusting software options, and performing data manipulation and presentation are discussed here.

GRAMS/32® Data Display and Main Menu

When Datamax-3D is run the software enters the GRAMS/32 Main Menu and spectral display. The top of the screen is reserved for the function menus and the icons for various software options. Information on most of the support functions (e.g. File options, View options, Peak Picking, Arithmetic functions, and spectral presentation) may be found in the GRAMS/32® Users Manual. Data collection and special features for the Spex 3D are discussed in this chapter. A brief description of these menus is listed below.

![Figure V-1: The GRAMS/32® Menu and Toolbar in Datamax-3D](image)

**Main Menu Options in GRAMS/32®**

The main menu options and their sub-options may be selected by clicking on them graphically or by hitting the underlined hot-key while simultaneously pressing the ALT button.

- **File**
  - Contains options for loading, saving, import and export of data files.

- **Edit**
  - Functions for editing spectra and view.

- **View**
  - Options for choosing different views for 3D acquisitions including Overlay,
    - 3-dimensional line, 3-dimensional image, and print views are offered here.
    - Menu choices for creating and editing views are also available.

- **Collect**
  - Offers the 3D_CCD menu option to commence data acquisition with the
Acquiring Data FL-3D with DataMax-3D

3D spectrofluorometer. More details on this option are found later in this chapter.

Peaks
Peak marking and related options are included in this menu.

Search
GRAMS/32® data archive and library options are offered in this section.

Arithmetic
Arithmetic functions, macro executables and Array Basic programming are accessible in this menu.

Options
Software options and user settings are in this menu.

Help
Help file information.

The GRAMS/32 manuals contain detailed information on all of these menus, with the exception of the Collect menu which is discussed later in this chapter.

Icons on the Toolbar in Datamax-3D Software

The toolbar buttons, from left to right are:

2. X-Axis Zoom  11. Back Peak
4. X&Y Axis Zoom  13. CCD Acquisition Parameters
5. Slider Zoom Bar  14. 3D Line View
6. Autoscale Trace  15. 3D Image View
8. Center Trace  17. Lock Scale
9. Page Up

Refer to the GRAMS/32® manual for information on these options.
FL-3D Experiment Settings

Selecting the Collect3D_CCD menu within GRAMS/32 opens the data collection section of the Datamax-3D software. The system will establish communication and initialize the CCD head for less than one minute.

There are several experiment options available in the CCD Experiment Settings.

Scan Parameters

CCD Area and Resolution

The rectangle at the top of the instrument, as well as the start and end (X Start, X End, Y Start, Y End) entries underneath allow you to specify the pixel or wavelength area of the CCD which will be used for the acquisition. To graphically select the region to be covered, simply click and drag a rectangle which encompasses the region from which you wish to acquire. Otherwise, type in the X and Y values at the appropriate prompts. For the FL-3D, the maximum pixel coverage is 1-512 on the X axis (250-750nm), and 100-412 on the Y axis (250-550nm).

There are two slide bars and text entries for the Resolution for the acquisition. For CCD acquisitions this is also called binning. Binning is the summation of charges for adjacent pixels in the readout register of the CCD controller. A lower number for these entries yields better resolution, while more binning will improve signal to noise and reduce the time per acquisition.

CCD Units

There are two choices for units to be used with the Spex 3D. Pixels, or the actual detector elements themselves, may be used as the excitation (Y) and emission (X) axes for the scan. Selecting Nanometers converts the data from pixels to the actual excitation and emission wavelengths. In order to perform this, each FL-3D instrument has its own spectral calibration files created at the factory to properly calibrate the instrument. For more information on this procedure for calibrating the CCD detector, please refer to Appendix D: Spectral Calibration Procedure for the FL-3D.
Once you have completed an acquisition you cannot convert the data between nanometers and pixels units. Therefore, please take the acquisition with the units you desire to work with.

For both options the Z-axis for the data is the CCD signal in photon counts. The dynamic range of the CCD chip for a single accumulation is 16 bits or 65535 counts.

**Exposure Time**
The exposure time setting has a slider bar and text entry box. The exposure time for the CCD chip may be set from 0.001 – 60 seconds. Shorter exposure times may be used for strongly fluorescent samples, and allow for rapid acquisition times. Longer exposure times should be used for weaker samples to take advantage of the dynamic range of the CCD head. Longer exposure times will also yield better signal-to-noise ratios for weaker fluorescent samples.

**Accumulations**
The number of accumulations is equivalent to the number of EEM's to be run for your sample. Accumulations are summed together and stored as a single datafile. The signal to noise for an acquisition will improve by the square root of the number of accumulations taken.

**Acquisition Modes**
There are two modes, Preview and Scan, for acquisition on the Spex 3D. **Preview** may be used to observe the EEM for a sample when setting up an experiment, or to simply monitor the fluorescence of a sample. **No data is saved in preview mode.** It is intended for performing preliminary measurements and checking instrument settings prior to running a Scan.

**Scans** are acquisitions which require a Datafile to be specified in order to save the spectral data. This data may be displayed in Image mode or Line mode by hitting the desired toolbar button before, during or after the acquisition. All scans are saved with an .SPC (spectra) extension.

**Slits**
Fixed slits are the only items not controlled by the software. Two sets of three different slit widths come with the FL-3D instrument, 0.05, 0.10 and 0.25mm. Each set is labelled X for excitation and M for emission. Excitation slits should be inserted with their labels facing towards the lamp housing. Emission slits should be inserted with the labels facing the sample compartment.

Choose the slit widths appropriately so that the emission signal will be within the linear range of the detector and will yield an acceptable resolution for the measurement of your sample.
Options

This button opens the *Options* menu which contains the option to load saved parameters, by loading an old acquisition data (.SPC) file or load default parameters. All acquisitions contain their parameters in their *File Information\Audit Log Trail.*

Additionally, the temperature of the CCD chip is also displayed in this window. Generally, it takes a few minutes after starting the CCD-3000 controller for the CCD to cool to below 250K. The software should have been started after the CCD was cooled below 250K so that the proper CCD initialization was performed by the software.
Running a Sample or Unknown

After a sample is placed in the sample compartment the procedure for taking an acquisition may be performed as follows:

1. Go toCollect3D_CCD
2. Define an acquisition with one (1) accumulation across the entire usable region of the CCD. Set the acquisition for nanometers. Set the resolution, or binning, to 4 pixels X 4 pixels. Set the exposure time to a mid-range value of around 0.5 seconds.
3. Run this acquisition in Preview Mode.
4. Select your region of interest from the full EEM. Retake a preview acquisition.
5. Judging from the data that results optimize the exposure time, binning and slits settings to take advantage of the resolution available and dynamic range of the CCD detector.
6. Take an acquisition in Scan mode using a suitable amount of accumulations for the required signal-to-noise for your acquisition.

Data Display and Manipulation Options

There are several options for how data can be displayed and processed. Some of the more popular options are discussed below. For explanations of all file manipulations available, please consult the GRAMS/32 Users Guide.

All acquisitions taken by the Spex 3D are saved a multifiles. Multifiles are the complete EEM's stored to disk. For processing, these files may be manipulated, extracted and imported or exported.

3-Dimensional Views

Datamax allows for several different types of three-dimensional plots to be available for the user. Wire frame, Hidden line and no-hide 3D trace views are available. Also, contour plots or image displays are available for data display. Data may be extracted from the 3D multifiles into single excitation, emission or synchronous traces through multifile utilities in the software. These individual spectra may be sorted through with overlaid, paged or stacked views. Finally, there is a projected map 3D view which combines a 3D and a contour plot.
Extracting Data by Customizing Views

GRAMS/32® within Datamax-3D allows you to create and save your own View files (.VW) in order to customize and manipulate the data presentation.

To extract data you should be using a contour plot or projected map. Reduce the size of this spectral view so that it fits in the left half of the viewable screen. Right click on the blank portion of the trace and select Add Object Spectrum or Chromatogram Trace. Then drag the ruler to form a rectangle for the size of the trace you want to add. When this is done, the software will ask for which type of trace to add. Choosing Paged will set up an emission scan extraction from the EEM. Choosing Extract CGM will set up an excitation scan extraction. When the trace is set up in the view, you may select an extraction by clicking on the trace on the contour map. This will send the extraction to the trace. Single clicking with the mouse extracts to the Paged view, while double clicking extracts to the Extract CGM view. If you do not see the extraction, click on the extract trace and autoscale the trace.

There is also a pre-defined extraction view called 3Dextract.VW that can be loaded from within the View menu. A complete procedure for creating and saving a view for extractions is detailed in the tutorial in Chapter VII.
**Spectral Extraction (Multifile Utilities)**

EEM's that are collected on the Spex 3D can be split into an array of single files or have a specific file or truncated file extracted from them. This is done using a macro (.AB) program called MFUTILS.AB or **Multifile Utilities**.

**Multifile Utilities** in the **Arithmetic Menu** provides several options for multifile manipulations. If you want to split a Spex 3D acquisition into single emission scans, choose *Split into Singles* to do just that. You must indicate if you want to write over the original filename or create a new one. Each scan will be sequentially numbered and offset per your settings.

*Figure V-5: Multifile Utilities*

To split a multifile into excitation scans, select *Rotate Multifile 90°* then *Split into Singles*. The multifile axes will rotate 90 degrees, then the excitation files will be extracted just as for the emission scans above.

*Figure V-6: Grams Multifile Operations*

**Arithmetic Functions and Multifiles (EEM's)**

There are several arithmetic functions available in GRAMS/32 which may be applied to the spectral data taken with the Spex 3D. Some of these functions may be applied to the entire EEM, while others may only be used on traditional X-Y spectrum files.

When working with arithmetic functions on EEM’s or multifiles in GRAMS/32 the function parameters setup will display a 2-D spectrum. When you have finished entering parameters the software will ask whether the function is to be applied just to the spectrum displayed or to each subfile of the EEM. Therefore, you can work with each subfile individually or manipulate the entire matrix at once.
When applying one multifile to another (e.g. EEM #1 subtracted from EEM #2), both multifiles must be of the same size and data density. The pixel binning, matrix size and wavelength units must be identical between the two multifiles for them to be manipulated properly. The number of accumulations and integration time for an acquisition do not have any bearing on this limitation.

Functions in GRAMS/32 which may not be used for multifiles will inform you as such if you should attempt to use them.

This chapter explains the procedure and options for acquiring spectral data using the Spex 3D. GRAMS/32 post processing functions are also explained. Try out the various experimental and data manipulation options available in the software on a few test samples. You may wish to perform the tutorial scans in Chapter 7.

When you have achieved a sense of comfort with the operating system, software and options start working with your own samples. While the information presented in this chapter is suitable for setting up and executing an acquisition, the next chapter will provide insight on optimizing the data which is collected.
CHAPTER VI

Optimizing Your Results

Once you start collecting data with the FL-3D spectrofluorometer, the next concern will undoubtedly be optimizing these results so that they are both accurate and publishable. This chapter is devoted to providing a variety of techniques to improve the data that is collected and help to present that data more appealingly.

Optimizing data should not only refer to the quality of the data collected, but also the time required to collect that data. Minimizing the time required for collection as well as increasing the quality of the data is a strong consideration for the chapter.

Methods for Optimizing Data During Acquisitions

There is no better time to think about optimizing the data that you will collect than before the experiment has begun. Then sections below discuss various techniques and considerations that should become part of approach to taking measurements with the FL-3D spectrofluorometer.

Setting up the Measurement Properly

Naturally, in order to obtain the best data, the instrument and the scan must be defined to allow for the best data collection. This section will explore the various instrument and software settings for an experiment and how to optimize them.

CCD Units (Spectral Calibration)

First, and foremost, you should acquire EEM's using nanometers as the CCD scan units. This converts the CCD data collected from pixel positions on the detector to the appropriate corresponding wavelength units for the EEM. Please refer to Appendix D for more information on the spectral calibration procedure that is run for every FL-3D instrument at the factory.

Collecting EEM's using pixels will show a qualitative view of the EEM, but it will not have any quantitative correspondence to the excitation and emission wavelengths of the data. You may collect in pixels to help locate your region of interest for measuring an unknown, while using preview mode, but when taking scans it is recommended to always use nanometers.
Resolution (Binning)
In the experiment setup there is an option to set the X and Y Resolution. In effect this sets the number of pixels that are summed together to form a single data point. But, since there is a wavelength spread across each pixel in both the X and Y axis (emission and excitation, respectively), the more pixels that are summed, or binned together, the lower the resolution of the acquisition. For the FL-3D data collection system, the more data points there are, the longer it takes to process the acquisition. The only way to reduce the number of datapoints is to decrease the resolution (by increasing the values for X and Y resolution).

You should set the instrument to collect with at least the minimum resolution required to observe and separate the spectral features that you desire to see in the data. If the resolution is set too low (e.g. 50nm in both the excitation and emission axes) then you will not see any spectral features. On the other hand, if you set the resolution finer than the experiment requires, you will still see the features that you were looking for (and maybe some others you didn't expect to observe). However, time will be wasted collecting the extra data and hard drive space will be wasted saving these spectra, when the resolution is set finer than required.

Spectral Coverage
The spectral coverage refers to how large an EEM you need to collect. The instrument has the capability to collect an EEM with excitation from 250-550 and emission from 250-750nm. Collecting too small a spectral range will obviously leave you with incomplete data. Collecting the entire EEM available on the instrument for every sample will be a waste of time and hard drive space.

When you are first looking at your sample, collect the full EEM available in Preview mode using the highest resolution of the instrument. Then find your particular region of interest, using the preview scan as a guide to set up your wavelength limits for your scans.

Finally, define the spectral coverage for the scan, and run the instrument in Scan mode.

Signal Levels
Getting the best data requires the best signal-to-noise. There are three settings which allow you to adjust the overall signal level to within the optimal range (20,000-60,000 cts for your highest peak in an EEM). These are the exposure time and accumulations settings in the scan definition, and the slits placed in the excitation and emission spectrographs. Later in this chapter, background intensity corrections are discussed. They can be used to reduce the background noise levels in the measurement.

Exposure Time
The exposure time is the amount of time that the CCD detector has signal incident upon it for a single accumulation. The amount of signal and the signal-to-noise is directly linked
to the exposure time setting for the acquisition. When setting the exposure time, remember that the dynamic range of the instrument is 0 – 65,535 counts.

You should set the exposure time to collect between 20,000 and 60,000 counts per accumulation at the highest peak level on the EEM.

**Slits**

The slits in the FL-3D instrument provide a trade-off between improving signal levels versus improving resolution. Three different slit widths are provided for both spectrographs in the instrument, 0.05, 0.1 and 0.25mm. The smaller the slit, the better the resolution, but the less signal that will pass through the system.

Choose the slits to use appropriate for the particular sample being measured. As with the **Resolution** setting, do not use slits with wide widths when you are trying to resolve a spectral feature. On the other hand, if you are simply looking for a relatively broad peak, the use wider slits to make data collection easier and faster.

**Accumulations**

The number of accumulations for an acquisition is the number of times you collect the experiment defined. Each accumulation is summed together and is stored as a single EEM. The advantage of using multiple accumulations is that it allows you improve the signal-to-noise of the measurement by a factor of the square root of the number of accumulations.

**Background Intensity Corrections**

Background intensity corrections are used to eliminate the background signal caused by the detector or by the solvent used for the measurement. Use of these corrections is vital to improve the signal-to-noise of the measurement.

**Dark Offset**

Enabling the dark offset option in the experiment allows the FL-3D to automatically subtract off the dark current from the scan data. For the FL-3D system this can remove between 1,000 and 4,000 counts of electronic and thermal noise from the CCD detector.

When adjusting the settings for overall signal levels, please remember that the dark counts of the instrument will reduce the apparent saturation level of the detector. So, if you are using dark offset to subtract off 3,000 counts from the scan, you will observe saturation of the detector if your samples signal exceeds 62,535 counts on the display.

**Blank Subtraction**

Blank subtraction is the technique of subtracting an identical acquisition of only the solvent used for a sample measurement from the actual sample acquisition.
To use this technique, set up and run the experiment for your sample (after you've optimized the settings). Then run the same experiment for a blank sample containing just the solvent. Go into the *Arithmetic* menu, select *Functions* and subtract the blank scan from the sample scan, then click *Add New* and finally, save the results.

This will remove the solvent contribution to the EEM as well as remove the dark counts from the CCD detector. *Do not use Dark Offset when you intend to employ Blank Subtraction or else you will subtract off the dark counts twice.*

**Proper Sample Geometry**

Setting up the sample properly can be the difference between excellent results and no results. Whether you are using aqueous solutions, turbid samples or solids, careful consideration of the proper geometry for measuring the sample will result in much more valuable data.

The most common types of samples which the FL-3D measures are aqueous samples. The samples are dissolved in a solvent and held in a cuvette container for measurements. Use Square or round cuvettes with the FL-3D system.

**Cuvette Materials**

Cuvettes are made from several different types of materials. The most common are acrylics, glass and fused silica (quartz). The difference between these materials are in cost, reactivity, and wavelength response. The less reactive and lower fluorescent the material the higher the cost.

Acrylic cuvettes are the most inexpensive and may be used for water and alcohol based samples which are excited in the visible. They tend to react with organic samples and can leach into other samples. Also, acrylic cuvette exhibit fluorescence when excited in the ultraviolet.

Glass cuvettes are much less reactive than their plastic counterparts. They will fluorescence in the UV (below 350nm), though not as much as acrylics.

Finally, fused silica or quartz cuvettes are the most expensive since they are relatively inert to the samples they contain. Also, quartz will only fluoresce below 200nm which is outside the usable range of the instrument. Whenever possible, it is advisable to use cuvettes made from this material.

There are other cuvette materials which you may find a desire to use. Most cuvette manufacturers are happy to explain the pros and cons of each material that they market.

**Microcells**

In addition to different cell materials, there are also different cell volumes available. The most common size for cuvettes is the 1cm square 4ml cuvette. The sample chamber is designed for this size cuvette. Using an appropriate adapter, you may also use smaller volume cells for when a ‘large’ volume of the sample is not available. We offer 1ml,
250μl, and microvolume flow cells as accessories for the FL-3D. Make sure when using a microcell that the entire excitation spectra (8mm of height at the sample) is incident on the sample, otherwise your EEM's will be compromised.

**Cuvette Preparation**
Sample cells should always be cleaned thoroughly before use to help minimize background contributions. The recommended cleaning procedure for fused silica and glass cuvettes follows.

**Cleaning:**

1. **Soak the cuvettes for 24 hours in chromic acid, which cleans the cuvettes.**
2. **Rinse with deionized water.**
3. **Prepare a cleaning solution by adding 20 pellets of potassium hydroxide to 100ml of HPLC-grade methanol.**
4. **Soak the cuvette in the potassium hydroxide solution for 5 hours.**
5. **Rinse well with deionized water. (This removes chromium ions, which can quench fluorescence.)**

**Important:** Soaking the cuvettes in the potassium hydroxide solution for a longer period causes etching of the cuvette surface, which results in scatter problems when the cuvettes are used.

6. **Soak the cuvettes for the last day in concentrated nitric acid.**

After cleaning,

7. **Store cuvettes in nitric acid until you are ready to use them and rinse them with deionized water before use.**

**Concentration Issues (Inner Filter Effect)**
The concentration of the solution used is critical to performing good measurements. The fluorescence yield and concentration of a sample determines how much light is emitted. Therefore, for small concentrations, the higher the concentration the more fluorescence which is emitted. However, a high sample concentration results in the Inner Filter Effect. This is the absorption of excitation light and/or reabsorption of the emission light by molecules in the sample not being observed. The higher the concentration, the larger the effect on the observed fluorescence intensity.

The inner filter effect is not seen on optically dense samples viewed on their front surface as absorption takes place on only the outer surface of the sample which is the point of observation.
Solid Samples

Solid samples are usually mounted in the Model 1933 Solid Sample Holder, and the fluorescence is collected from the front surface of the sample at 45° (see Chapter VIII, Accessories). The method of mounting the solid sample depends on the particular sample. Thin films and cell monolayers on coverslips can be placed directly in the solid sample holder. Minerals, crystals, vitamins, paint chips, and similar samples are usually ground into a powder to make a homogeneous mixture. The powder is packed into the depression of the solid sample holder. If it is very fine or resists packing (and therefore falls out when the holder is put into its vertical position), the powder can be held in place with a thin quartz coverslip or blended with potassium bromide for better cohesion.

Solid samples, such as crystals, can sometimes be dissolved in a solvent and analyzed in solution. Solvents, however, may contain organic impurities which fluoresce and mask the signal of interest. For this reason, use high-quality, HPLC-grade solvents. If background fluorescence persists, recrystallize the sample to eliminate organic impurities and then dissolve it in an appropriate solvent for analysis.

Sample Conditions

Sample conditions refer to the physical and chemical environment of the samples being measured. This section discusses stirring and temperature control as examples. These and other possible environmental factors should be considered before the experiment, in order to ensure the best results.

Stirring

For reproducible results, some samples may require physical agitation. For example, proteins, cell membranes, and cells in solution need constant stirring to prevent settling. We offer a sample stirring accessory and there are various types of stirring bars available for a wide variety of cuvettes and other containers.

Heating and Cooling

Other samples are temperature sensitive and must be heated or cooled to ensure reproducibility in fluorescence signals. Proteins, cell membranes, and other complex molecules show a greater ability to rotate when heated and, thus, exhibit different fluorescent characteristics. Use heating and cooling to hold your sample in a steady state with respect to temperature or to create a temperature curve using a programmable temperature bath.

Sample Effects

This section discusses two special sample effects in fluorescence measurements, photobleaching and quenching. Both can cause difficulties in fluorescence studies.
Photobleaching
Photobleaching is reduction of fluorescence output from the sample due to excessive and prolonged exposure to excitation light. It can appear as a gradual or rapid decrease in fluorescence intensity over time. To help prevent photobleaching, keep the excitation shutter closed between acquisitions and use the appropriate shutter modes available in time base scans and CWA acquisitions. Also, using smaller excitation slits combined with wider emission slits can reduce the intensity of light that is incident upon the sample while maintaining the total fluorescence observed.

Solvent Quenching
The choice of solvent for an aqueous sample is very important. Some solvents will exhibit luminescence or Raman scattering in the region of interest for your sample. Other solvents may quench your sample by providing a pathway for non-radiative decay or energy transfer and a shorter fluorescence lifetime. Make a careful selection of solvents to avoid this type of effect upon the samples you measure.

Other Solvent Effects
Please consider other effects that the solvent can have on the sample. For example, many samples possess luminescence that is pH sensitive. Using the wrong solvent can result in apparently erroneous results, throwing an assay into a tailspin. A little time spent investigating the sensitivity of the fluorescence of a sample can result in a great deal of frustration saved after the assay results are processed.

Spurious Data
There is nothing worse for a spectroscopist than being tricked by spurious data or having those extra peaks overlapping their region of interest. The two main classes of artifacts result from Rayleigh and Raman scatter, and from second order effects of the optically dispersive grating elements of the spectrographs.

Some suggestions for how to minimize these effects and avoid them are explained below.

Scatter bands and phenomena
Rayleigh scatter, or excitation scatter, is observed when the excitation wavelength equals the emission wavelength. This band will be routinely observed on the 3D as a diagonal line on the EEM with a slope of 1. The Rayleigh band is more intense at lower wavelengths being theoretically proportional in intensity to $1/\lambda^4$. Additionally, this band is observed in second order effects as well.

Raman scatter, is a solvent effect which occurs at a fixed energy red-shift from the Rayleigh band. It is much smaller in intensity, but is often mistaken for fluorescence from the sample. Documentation of various Raman scatter wavelengths are available in texts on Raman spectroscopy. For water, this shift is $3380 \text{ cm}^{-1}$. 
An easy way to verify if a peak is a scatter peak is to see if it shifts with excitation wavelength. A peak that shifts with excitation wavelength is almost always the result of scatter.

**Second order effects**
Diffraction gratings separate light using finely etched grooves in the surface of the optic. A side effect of this dispersive process is that multiple orders (integer multiples) of a particular wavelength are passed through the system. Therefore, a peak at 300nm would also be dispersed through the system at 600nm, 900nm and so on. Additionally, the band would be seen at 150nm, 75nm, etc.

The solution for removing these bands at these wavelengths is that they still have the energy of the true wavelength. Therefore, optical filters may be used to remove these multiple orders.

**Optical Filters**
Optical filters may be used on the FL-3D instrument. They will have a significant effect on the EEM's being collected. Consider the effects the filters chosen will have on the EEM before setting the spectral coverage. For example, if you are using a 400nm cut-on filter, then all of your data below 400nm will be invalid, and should not be collected.

**Spectral Correction**
Spectral intensity correction of the FL-3D is currently not provided with the instrument. This would involve correcting for the wavelength response of the FL-3D instrument and the CCD detector across the entire usable EEM.
Methods for Optimizing Data in Post Processing

After the data is collected there are also some common techniques for improving the quality of the data as well as for perfecting the data presentation. While some post-processing functions may be applied to the Grams multifile (EEM), most of these techniques must be performed on the individual subfiles of the EEM.

Views (Paged, Overlaid, 3D Line, Contour Plots, custom)

After the data has been collected, Grams/32c offers a variety of different views to improve the presentation of your data. In addition, these views can assist you with other post-processing functions, such as data extractions and peak marking. You can refer to the Grams/32c Users Manual for more detailed information on these views.

The 3D Image View, also known as a Contour Plot, is very useful for analyzing the entire excitation emission matrix. Use the XYZ Settings to set the intensity scaling and the wavelength area, as well as optimize the number of contours and interpolation for the display of the EEM.

3D Line Views, which include a few different options are useful for resolving various peaks in the spectra, and are quite nice for output to printer devices. However, when viewing the entire EEM, it is recommended to use the Image View.

Paged and Overlaid views are used to view the individual subfiles which make up the spectral multifile (or EEM). You can use the page up and page down icons on the Grams/32c toolbar to flip through the individual spectra of the multifile. These views are good for examining individual excitation or emission spectra within the EEM. You may save these subfiles individually by using multifile utilities or by using the 3Dextract view defined in the software.

Custom Views may also be designed, stored and reloaded by users. This allows you to detail you own specialized displays for data manipulation and data presentation. Text, graphics, and additional spectra can be added, edited or manipulated to display results in a publishable format that highlights your findings. Please consult the Grams/32c manual for all of the available options for creating your own views.

Data Extractions

Use the three data extractions available to help you isolate the individual spectra that highlights your results. Extractions are available on the excitation, emission or synchronous axes. Use excitation and emission extractions to pull out your peak positions within the spectra. Synchronous extractions are useful for separating mixtures of fluorophores.
Optimizing Your Results

**Arithmetic Functions**

Grams/32c supports a wealth of arithmetic functions for post-processing of the data. Most basic arithmetic functions may be performed on the multifile as a whole. Specialized functions can only be run on individual subfiles (extractions). You may be prompted to run a macro program called XY2EVEN.AB to interpolate the spectra to even X spacing. When spectral calibration is applied to the EEM, the resulting data does not possess even X spacing, requiring the interpolation routine to be run.

Use arithmetic functions to subtract off blank spectra, or to manually remove dark counts. Details on the functions available are found in the Grams/32c Users Manual.

**Smoothing Functions**

If you find that your data is noisy, there are smoothing functions available in the Arithmetic Menu of the Grams/32c software as well. You can use smoothing or curve fitting to help make noisy data more presentable.

Also, if you encounter any cosmic ray events, you can use a function called ZAP.AB to remove these unwanted spikes from the data.

**Peak Marking**

Peak Marking may only be run on individual subfiles of an EEM. After taking an extraction (or breaking into subfiles), you have a couple of options to set the sensitivities for peak picking and also for how the peak marks are displayed. Also, when run, a table of the peak values may be saved (.PKS extension) for use elsewhere for post-processing.

Use *Auto Settings* when you want to zoom in on the smallest peak that you wish to detect and mark. Clicking *Auto Settings* will then automatically set the various peak picking parameters for you. Otherwise, click *Settings* and manually enter the sensitivity, threshold or slope parameters that you wish to use for finding peaks. When complete, hit *Mark All Peaks* for the peaks to be marked.

**Exporting Data**

Many users want to export data to an external data manipulation and display program such as SigmaPlot® or Microsoft Excel®. Grams/32c supports a multitude of different data interchange formats for moving your data in and out of the software.
The data that you collect during an experiment may be optimized in several ways. This chapter explores several of the options available within the software for improving data collection and presentation.

The next chapter provides a tutorial for data collection as well as optimization, helping you get a feel for the user interface and functionality of the FL-3D spectrofluorometer.
CHAPTER VII

A Tutorial Acquisition

This section provides a tutorial acquisition of a fluorophore 'cocktail' for the Spex 3D. It is intended to provide 3D users with an example of experiment setup, data acquisition, processing of the measurement, and data presentation for an unknown sample. In addition, common questions asked, pitfalls encountered, and methods of optimizing these measurements are discussed.
Samples

A mixture of anthracene, perylene and fluorescein in methanol. These fluorophores and methanol are available through Aldrich or Sigma Chemicals.

The maximum absorption for each fluorophore should not exceed 0.1 Absorbance units in its region of interest. Our experimental data has the following absorptions for each fluorophore in the mixture.

- Anthracene ~ 0.1 A @ 355nm
- Perylene ~ 0.02 A @ 432nm
- Fluorescein ~ 0.01 A @ 491nm

**Important**

Most organic fluorophores and solvents are known health hazards. Please read the instructions contained on the packaging in the Materials Safety Data Sheets for these fluorophores and solvents before working with them.

In this tutorial, the samples will be mixed together to form a fluorophore 'cocktail' and scanned with the FL-3D instrument. However, you may also run the individual fluorophores for more practice with acquisitions using the instrument.

Place the combined samples in a stoppered 4ml fused silica cuvette. Seal the cuvette with parafilm. Glass cuvettes and acrylic cuvettes may provide a high level of background fluorescence throughout the ultraviolet wavelengths. The total volume of the samples should be at least 2ml so that the instrument can collect the entire spectra off the sample. In cases where lower sample volumes are available, microcells with adapters to the sample mount may be used.

Also, prepare a methanol blank to be used later in the experiment.

Theory

Anthracene, Perylene and Fluorescein are used for the acquisition since each fluorophore absorbs and emits at different wavelengths. Anthracene excites in the UV, between 280 and 350nm. Perylene excites between 350-400nm. Fluorescein excites at about 490nm. The samples also have different chemistry as is seen by their spectra. Anthracene and Perylene have very sharp vibrational bands which make them very nice examples for the mirror image rule. Fluorescein has a single primary excitation/emission pair and a high quantum yield.
Experimental Procedure

Summary

In this experiment you will take preview scans of the Anthracene, Perylene, Fluorescein fluorophore 'cocktail' (henceforward referred to as the cocktail). After preview scans are taken and system settings are optimized, stored scans will be acquired for the cocktail. After acquiring satisfactory data, you will proceed to use the different view, arithmetic, and display options to optimize the data presentation. Finally, the data will be exported in Microsoft Excel™ format.

Procedure

Setup and Preview Mode

1. Power up your system by first turning on the FL-3D instrument, then the CCD-3000 controller and finally your host computer and peripherals. When the Windows desktop comes up, run the Datamax-3D software.

2. Go to Collect Experiment / 3D_CCD and wait until the Datamax-3D acquisition menu appears.

3. Click on the Options button at the bottom right end of the menu. In this menu, verify that the CCD temperature is less than 253 Kelvin (-20°C). If not, wait until the CCD cools to below this level. Restart the software and return the Datamax-3D acquisition menu.

4. Set the X and Y limits for the maximum coverage allowed on the chip. You may select this by double-clicking on the CCD area field at the top of this menu.
5. Set the **CCD Units** to *Pixels*. Set the X and Y resolution to 1 pixel, the maximum resolution. You may use the slider bar for resolution or simply type it in the field provided.

6. Set the **Exposure Time** to 1 second. You may use the slider bar or type in your entry.

7. Set the **Accumulations** for one (1) accumulation during the acquisition.

8. Insert the 0.1mm slits into the excitation and emission spectrographs. The excitation slit is marked ‘X’ and the emission slit is marked ‘M’. The excitation slit fits on the left side of the instrument, while the emission slit goes into place on the top right side of the Spex 3D.

9. Remove the sample cover lid. Place the cuvette containing the fluorophore cocktail into the sample mount of the Spex 3D instrument. Observe how the diffracted excitation spectra passes through the cocktail along the height axis of the cuvette. This is the incident excitation which is paired with a horizontal emission spectrograph to collect the excitation/emission matrix for your sample. If the sample volume is too small, you will see the light pass through the sample cell without passing through the cocktail. Thus, it will not induce and spectral emission!

10. Replace the lid over the sample compartment.

11. Click on **Preview** in the Datamax-3D menu.

   You do not need to specify a filename for the Preview acquisition mode as data is not automatically saved when using this mode. This mode should be used only for previewing the data from your sample and settling on parameters for an acquisition that you will want to store or manipulate.

   The spectral matrix image for your data should be collected and displayed in a couple of seconds.

Now that you have acquired the data there are several options available for viewing it. In Grams/32 select the **3D-Image** view in the **View** menu and then hit the full scale toolbar button. The trace will appear as a contour plot of the excitation/emission matrix.

The vertical axis for the trace is the excitation while the horizontal axis is the emission. The units for this acquisition was set to pixels so these are the units used for this display. You may not toggle between units after an acquisition.

On the trace you should see spectral features following a staircase from the lower left corner of the contour map. The lower left spectral features are from Anthracene. The next step has the Perylene spectra. The final broad peak is for Fluorescein.
Customize the settings for this view by hitting **XYZ Settings** in the **View** menu. Set the minimum Y to 0. Increase the number of contours to 20 or 30. The view will increase the number of contour lines and reset the minimum Y for you. This is just a couple of the minor manipulations of data presentation that are at your fingertips with Datamax.

Select the **3D Line** view in the **View** menu to look at the data as a 3D trace representation. There are also 3D no-hide and 3D wire frame views available in **Trace Modes**.

Hit the **Scroll Lock** ("Hand") icon on the GRAMS/32 toolbar (the first one of the left). Use the "hand" to pull the axes of the three dimensional graph and alter the view of the spectra.

There are several options which may be varied for scans. You may change the number of accumulations, X and Y resolutions (binning), slit settings, and detector exposure time. Pixel resolution and slit settings directly impact on the resolution of the instrument. The exposure time, the duration of time that the CCD detector has signal incident upon it, determines the dark count and signal levels observed in the data. You will be asked to try varying these parameters in the next section of this experiment.

12. Verify that none of the data is saturated (equal to 65,535 counts for a single accumulation), and that there is good dynamic range in the data (maximum intensity is between 10,000 and 65,000 counts). If the data is not at the proper levels, return to the parameter definition screen and adjust the exposure time and/or slits. Retake Preview scans until the data is in the optimal signal range.

13. Vary the slit settings and repeat the Preview scans. Notice, how wider slits both increase the overall signal levels and decrease the resolution of the trace. Your choice of slit widths to be used for an acquisition should take into account both of these facts.

14. Try changing the number of accumulations from one to five. The system will now take five exposures as opposed to one. The data will be summed and displayed. Multiple accumulations may be used to help improve signal to noise.

15. Change the X resolution and Y resolution and repeat the Preview scans. Counting a group of pixels together as one data element is known as **binning**. It is used to improve the signal to noise in a measurement. But, in the FL-3D, area elements that are binned together represent wavelength elements counted together. Therefore, increasing the binning will reduce the resolution but improve the signal-to-noise. Additionally, increasing the binning will reduce the amount of time needed for data acquisition and display. Weigh your requirements for resolution, sensitivity and time when setting these parameters.
16. Finally, there is one more item which must be changed before entering Scan mode, the CCD Units. Change the units from Pixels to Nanometers. Now, the software will apply a conversion from the X,Y pixel position to a corresponding excitation/emission wavelength position.

Notice how the X and Y limits have changed from pixel positions to wavelength positions. Also, the resolution values have changed from pixels to nanometers. For most acquisitions, nanometers should be the CCD units used.

Take one or two more Preview scans and see how the data display has changed.

**Scan Mode**

Now that you have optimized the signal levels and other system settings you are now ready to acquire a Scan. A Scan is really the same as a Preview, except that you must specify a filename for the scan to be saved prior to the acquisition.

17. Return to the Parameter Definition Screen and click on Datafile. Use the Explorer to find the directory and specify the filename which you wish to save the scan as. All Spex 3D scans are saved with an .SPC extension. Filenames must be eight characters or less.

You may note that clicking on the filename in the display has no effect. You must use the Datafile button to specify the filename.

18. Check that you are satisfied with your parameters, then click the Scan button. The acquisition will now proceed.

19. When the scan is complete, go to 3D Image View and adjust the contour map to your satisfaction. You should notice the filename in the lower left hand corner of the view.

20. If the scan is satisfactory, go back to the parameter definitions, change the datafile to BLANK.SPC and repeat the scan on the methanol blank. Use the same parameters that were used for the fluorophore cocktail.

**Post Processing Functions in Datamax-3D (GRAMS/32) Software**

Now that you have collected some worthwhile data, we will now proceed to processing the data using the GRAMS functions available in the software. In the following procedure you will perform the following processing operations:

(A) Subtract the blank scan from the sample run (illustrating math functions on 3D multfiles).
(B) Display the scan in a variety of modes including the contour map, various 3D plots and the Overlaid view mode.

(C) Display the trace in the contour map, then add trace boxes to a new view to extract single excitation and emission spectra from the multifile.

(D) Split the multifile into single spectra by emission and by excitation.

(E) Export the Data to Microsoft Excel™ format by running DDEEXCEL.ABL, a macro program available on the Galactic Industries Website.

Subtracting off the Blank Scan
This exercise will serve as an example for applying math functions to a GRAMS multifile.

1. Select the fluorophore cocktail datafile by selecting it if its in memory or going to File/Open and loading the file into memory.

2. Click on Arithmetic/Functions. The function screen will come up with the first subfile of the cocktail multifile shown in the bottom trace view.

3. Within the Operation window set Function to Subtract Operand to Term File X K

4. At the Term File window click the Select button and select the blank scan which you ran for the methanol blank.

5. Click on the Apply button.

6. You should observe the arithmetic function being processed for the entire datafile. After this one subfile of the result will be displayed on the upper trace view.

7. Click OK. Datamax-3D will now prompt you to supply a new filename for the result file. Save this file with the name you wish to use.
Some arithmetic functions will give special prompts if they are to operate on multifiles. You will basically have the option to apply the function to all subfiles or to one the subfile being displayed. Also, there are a few arithmetic options which are not valid for datafiles, these will prompt you as such if you try to use them.

If you prefer to split your data into individual subfiles (single excitation or emission traces), you may also use these arithmetic functions and routines on the subfile data.

**Different Views for the Data**

All Views may be selected directly from within the *View* menu. Three are available directly within this window; *3D Line*, *3D Image*, and *Overlaid*. You may either select a view and the open datafiles or have a datafile open, change display views then autoscale the traces in memory.

![Excitation/Emission Matrix of Fluorophore Cocktail containing Anthracene, Perylene and Fluorescein](image)

*Figure VII-3: Excitation/Emission Matrix of Fluorophore Cocktail containing Anthracene, Perylene and Fluorescein*

Datamax-3D offers several views, in addition to those in the *View* menu, for interpreting and displaying spectral data. All trace view options are available in the *Trace Mode* menu within the *View* menu. This exercise will work with five of the available trace view modes. Five of these modes are for multifiles, *Hidden Line 3D*, *Wire Frame 3D*, *No Hide 3D*, *Contour Map*, and *3D Projected Map*. The last mode is *Overlaid* mode which may be used to flip through the individual 2D subfiles making up the multifile.
**Contour Maps (3D Image View)**

An example of a contour map for the fluorophore cocktail is shown above.

8. Go to the toolbar and select **3D Image View**. This view is a contour map of the CCD image. Open the blank subtracted fluorophore cocktail file and autoscale it in this view.

9. In the **View** menu select **XYZ Settings**. Try different settings for the number of contours and interpolation for the image. Generally, the more contours and interpolations the smoother the plot (and the more time required to process and display it). The plot above uses 8 contours and no interpolation.

10. Right click on the contour map and select **Edit Settings**. At **Line Style** go to the bottom of the list and select **Special Colors**. This option adds color intensity shading to all 3D views. It does require a bit of display memory, but is quite worthwhile, especially with color printers and displays.

11. Print out a copy of the contour map. Users with black and white printers should set the contour map for black and white mode with less contours as in the printout above. Users with color printers should user the default color mode or use the special colors mode. Make sure your printer settings are as desired (e.g. Portrait vs. Landscape, desired resolution, etc.).

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**No Hide 3D (3D Line View)**
This view is the default 3D line view on the Datamax-3D toolbar or the 3D Line View option in the View menu. No-hide means that all traces are shown superimposed on one another.

12. Now work on the blank subtracted cocktail datafile in this 3D view. Use the scroll lock option ('hand' toolbar icon) to change the perspective of the plot.

13. Right click on the view, select Edit Settings and set the Line Style to Special Colors. Go to XYZ Settings in the View Menu and set the number of contours to 8. Now the trace should have different color contours based on intensity levels.

14. Play around with the other manipulations for this trace.

Hidden Line 3D and Wire Frame 3D Views
These views are self-explanatory. For the Hidden Line 3D view, all data behind a peak from the perspective chosen is not drawn, otherwise it is the same as 3D no-hide. Wire Frame 3D is the same as Hidden Line 3D, except the data is fit together with a wire mesh.

15. Try out these views with the data, customizing the various settings available for 3D trace views.

16. When done you should try to print out hard copies of a couple of these view.

Overlaid View
The last view that will be covered in this tutorial is the overlaid view. When working with multfiles it is sometimes easier to flip through the individual emission spectra (or excitation spectra) to see the spectral shapes. Use Overlaid view on the View menu or in the Trace Mode menu to do this without having to extract the files first.

17. Once again, work with the blank subtracted fluorophore cocktail file. Select Overlaid View. Hit the Autoscale toolbar button. You should observe a two dimension trace like the one shown above.

18. Use the PAGE UP and PAGE DOWN keys to flip through the many subfiles of the cocktail multfile. Choose a file that has some interesting emission spectra. The one shown above is midway through the multfile showing the emission contribution from Perylene in the cocktail.

19. Practice optimizing the various settings for this individual subfile, and try to print it out. If you wish to save a certain subfile or do further work with it, you should extract the file from the multfile. That subject will be covered later in this chapter.

20. Go to the View Menu and select Table View. The subfile data is now displayed in spreadsheet format. You click on individual cells and change datapoint values or perform other basic operations. Return to the Overlaid View.

This is the end of the tour for the different views available in Datamax-3D for the Spex-3D. There are other views available including a Print View for printing 2-D spectra with thicker lines, and 3D Projected View, which combines a contour map and 3D-hidden line view.
The next section describes how to customize or create your own views in Datamax-3D. It will be used to create a view for extracting excitation and emission spectra from the excitation/emission matrix.

**Customizing a Trace View in Datamax**
**(and using it for Extraction of Excitation and Emission Spectra from the EEM)**

This exercise should prove quite useful for your research on the Spex 3D. You will take the blank subtracted fluorophore cocktail datafile and set up a view which will allow you to extract selected excitation and emission spectra to be printed, saved or exported. This view may be loaded later for use with future data to aid in data presentation and manipulation.

21. Open the blank subtracted fluorophore cocktail datafile.
22. Click on the *3D Image View* icon on the toolbar.
23. Click on the edge of the trace display for the contour, grab a corner tab for the scan and resize the plot to fit in less than half of the screen.

![Image](image-url)  
*Figure VII-6: Creating a View; Resizing the Trace*
24. In the empty space left on the view, left click on the mouse and select *Add Object / Spectrum or Chromatogram Trace*. Using the rulers icon drag out the area for the trace in one empty quadrant of space left on the view.

![Figure VII-7: Creating a View; Adding a New Trace](image)

25. When the *Trace Object Parameters* menu comes up, select *Paged* for the display mode. This creates a single spectrum view for the selected emission spectrum to be extracted to.

![Figure VII-8: Trace Object Parameters](image)

26. Go to the contour plot and single click on a desired point to extract an emission spectrum to the Paged trace.
27. Click on the Paged trace and hit the autoscale icon. You should observe the emission spectra for the point that was selected on the contour plot.

28. Create another new trace in the remaining open space left on the view, by again left clicking the mouse and adding another Spectrum or Chromatogram trace. Note: All traces are extracted as chromatograms in GRAMS/32. This gives the traces a .CGM extension when they are saved.

29. When the Trace Object Parameters menu comes up, select Extract .CGM for the display mode. This creates a single spectrum view for the selected excitation scan from the contour plot.

30. Go to the contour plot and double click on a desired point to get an excitation spectrum.
31. Click on the Extract .CGM trace and hit the autoscale icon. You should observe the excitation spectra for the point that was selected on the contour plot.

![Image of DataMax-3D software interface]

*Figure VII-11: Creating a View: A View for Spectral Extraction of Excitation and Emission Spectra*

Now, any time you single or double click on a point on the 3D contour plot (image) an emission or excitation scan, respectively, will be updated to the Paged or Extract .CGM trace display. Using this view you may manipulate and extract any spectra that is desired from the excitation/emission matrix.

If you wish to further customize this display (e.g. change axes labels, add text, etc.), please refer to the GRAMS/32 manual.

To save the view, open the View menu and select **Save As**. Then save the view with a filename different from the default views, but with a .VW extension. You may load the view later when you wish to work with your 3D files.

When you exit Datamax you will be prompted to save the extracted datafiles as individual spectra if you desire.
Splitting Multifiles into Single Spectra by Excitation or Emission

Often users prefer to work with single excitation or emission spectra as has been traditionally done on spectrofluorometers. With the 3D system, the entire excitation/emission matrix is collected in one rapid acquisition. To work with the single spectra you can create the view to extract a preferred spectrum as detailed above or you may use Multifile Utilities in Datamax to extract files into singles to be worked with individually. This section of the tutorial will take you through extracting a multifile into single emission scans or single excitation scans using the Multifile Utilities.

32. Go to the Arithmetic menu and select Multifile Utilities.

33. The multifile utilities menu will offer several options for working with multifiles. First, we’ll split the multifile into single emission spectra. Select Split into Singles.

![Figure VII-12: Multifile Utilities](image)

![Figure VII-13: Grams Multifile Operations](image)
34. Follow the directions for choosing filenames, then choose to create the maximum suggested number of individual files (indicated in the menu). The software will now perform the extraction for you with the files numbered sequentially in .SPC format.

35. Go to a paged or overlaid view and browse through some of these files.

36. Splitting into single excitation spectra requires an extra step. First, select **Rotate 90 degrees**. This will change axes for the trace. Supply a new filename for the rotated multifile which will be created.

37. Now, simply choose **split into singles** again. After rotating the multifile 90 degrees, the extraction will be done by excitation spectra.

38. Review these spectra in an overlaid trace view mode.

Consult the GRAMS/32 manual for more information on the other multifile utilities available in GRAMS.

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**Exporting Data from Datamax to an Excel™ Spreadsheet**

Normally, only 2-D spectra may be exported into a spreadsheet. However, there is a file available on the Galactic Industries website, called DDEEXCEL.ABL, which exports an entire multifile to Excel™ directly. In this exercise, you will download the information from the Galactic website, then use the macro program to export the blank subtracted fluorophore cocktail EEM to Excel™.

39. Log on to the Galactic website, [WWW.GALACTIC.COM](http://WWW.GALACTIC.COM) and proceed to the downloadable program section. Download the DDEExcel programs available on the website and place them in your GRAMSN directory (or the directory where the software is installed). This website contains information on GRAMS/32 and may be used as a resource for information on the software.

40. Enter the software. Run DDEExcel and follow the instructions given for exporting the blank subtracted fluorophore cocktail file.

41. Go into Microsoft Excel™ and review the data. ‘

This macro is an effective tool for exporting multifile data out of GRAMS/32. If you are interested in creating your own macro programs please refer to the GRAMS documentation.

This completes the tutorial for the Spex 3D spectrofluorometer.
In this tutorial you have set up your sample, optimized system settings and collected data on a fluorophore ‘cocktail’. In post processing, different views were used to observe and manipulate the data. Also, math functions were applied to the data.

Finally, you should have done some data extraction and exported multifile data to a spreadsheet. Hopefully, you have certain level of proficiency with the system. Try taking data with more trial samples until you are fully comfortable with using the Spex 3D.
CHAPTER VIII

Accessories

This section will describe optional accessories for the Spex 3D. These accessories enable you to customize your Spex 3D for specific applications. For information about these and new fluorescence accessories, please call ISA or your local representative.

The following list represents the accessories that are available for the Spex 3D spectrofluorometer. Contact Instruments S.A. or visit our Webpage at HTTP:\WWW.ISAINC.COM to find out if any new accessories are available.

For additional information or product literature on any of these items, contact Fluorescence Service.

<table>
<thead>
<tr>
<th>FL-3D ACCESSORIES (ALPHABETIZED)</th>
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<tbody>
<tr>
<td>ITEM</td>
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<tr>
<td>LAMP, Short Xenon Replacement, 75W Ozone-Free</td>
</tr>
<tr>
<td>SOLID SAMPLE HOLDER</td>
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</tbody>
</table>
Model 43.311.302 Xenon Lamp
The 75W xenon lamp for the Spex 3D provides a broad continuum of light for the excitation of fluorescent samples from 250nm to above 1 micron. The lamp is ozone free and does not require any additional venting other than that provided by the instrument. These lamps have an average lifetime of 400 hours.

Model 1933 Solid Sample Holder
The solid sample holder provides a stable mount for solid samples, thin films and powder samples. The sample holder may be rotated easily for proper system alignment. The holder consists of a standard base, with a rotatable bracket with spring clip and power sample block (or backing). The sample block may be packed with a powder for analysis, or it may be used as a backing for a thin film or gel sample.

This list of accessories is current to the date of publication. However, conscious of the ever-changing needs of researchers, Instruments SA continues to design, manufacture, and improve accessories for its product lines. To find out what other accessories may exist, feel free to contact the Fluorescence Group or your local sales representative.
CHAPTER IX

Maintenance

Your Spex 3D spectrofluorometer requires little maintenance. The outside panels may be wiped with a damp cloth to remove dust and fingerprints. The lamp is the only component that requires routine replacement. It is recommended to log the hours used on the lamp for proper maintenance.

Additionally, it is recommended to verify the instrument calibration about once per month by performing an acquisition of a standard fluorophore such as Fluorescein or a scatterer such as glycogen.

Xenon Lamp

Obtaining good spectral results is contingent upon the condition of the Xenon lamp. The lamp is a 75 Watt Xenon short arc. It does not produce ozone. After 400 hours of use, the lamp output decreases significantly, indicating that the lamp should be replaced.

_Important_

Replacing the lamp within the specified time may prevent a failure of the Xenon lamp. Xenon lamps are known to explode from gross overuse. Please replace your lamps regularly, or call our Fluorescence Service Department for assistance.

Replacement

![Figure IX-1: 75W Ozone-Free Short-Arc Xenon Lamp](image)

The lamp is held in place using a spring-loaded mount allowing for thermal expansion. This mount contains adjustments for the lamp height and distance from the ellipsoidal mirror. When following the procedure detailed below, minimal realignment of the 3D should be required for a replacement Xenon lamp.

Read all the packing material including instructions and precautions before attempting to insert the lamp into the lamp housing. Do not remove the protective cover from the
replacement Xenon lamp until instructed to do so. Remove the AC power connections to the Spex 3D instrument prior to replacing the lamp.

![Caution]

**Caution**

Xenon arc lamps are an explosion hazard. Wear goggles to protect your eyes from UV radiation as well as from any debris in the event of an explosion. In addition, wear protective clothing when handling the lamp.

The lamp power supply should not be connected to an AC power line while handling lamp leads. Lethal high voltages may exist.

Never look directly at the xenon arc or its reflection. Intense radiation can permanently damage eyes.

Do not touch the ellipsoidal mirror or the surface of the lamp. Fingerprints will be burned onto these surfaces when the lamp is ignited.

Read and follow the cautions presented below whenever using or handling Xenon lamps.

**To replace the Xenon lamp in the Spex 3D:**

![Figure IX-2: 75W Xenon Lamp in Plastic Shipping Cover]

1. **Unpackage the new Xenon lamp and leave it lying in half of its protective cover in its shipping box. Save the other half of the protective cover for the old lamp to be placed in.**

2. **Note the orientation of the old lamp prior to removing it. In particular, note where the bubble faces. Then new lamp should be installed in the same orientation.**

   Remove the Spex 3D cover by removing the screws running around the top of the 3D instrument. Gently, lift off this cover and sit it nearby in a safe location.
3. Remove the old Xenon lamp, with the anode and cathode connections still on, by gently pushing the lamp downwards till the anode can be removed. Then, lift the lamp out of the socket for the cathode.

4. Remove the brass fittings which attach the bias connections to the lamp using an 1/16" set screw.

5. Place the old lamp in the half of the protective cover set aside for it.

6. Slip the brass fittings onto the lamp and secure them to the lamp with a 1/16" allen wrench.

7. Place the new lamp into the lamp mount by putting the cathode into the bottom of the lamp mount, then gently pushing downwards and slipping the lamp into place at the anode. Set the lamp so that the bubble faces towards the baseplate (as it was with the older lamp).

**Important:**
The anode is larger than the cathode and the small bubble on the envelope of the lamp marks the anode side.
8. Cover and seal up the old lamp in the packing materials for that came with the new lamp. Follow the instructions sent with the Xenon lamp for proper disposal instructions.

9. Replace the Spex 3D cover taking care not to strike the lamp mount with the cover. Replace and tighten the screws for the instrument cover.


11. Start up the Spex 3D system by turning the rocker switch, in the rear of the Spex 3D to the on position. The lamp should arc in a couple of seconds.

   If the lamp arcs properly, proceed to the section for lamp adjustment instructions if further alignment of the lamp is necessary.

   If the lamp fails to arc, shut off power to the Spex 3D immediately. Disconnect the AC line cord from the Spex 3D and recheck all connections. If the lamp still will not arc, please consult the troubleshooting section in the next chapter of this manual and/or contact Spex Fluorescence Service.
Spectral Calibration Verification

The Spex FL-3D spectrofluorometer should have its spectral calibration checked roughly monthly to verify that it is performing properly. The instrument is composed of two fixed grating spectrographs along with a fixed CCD emission detector, and, as such, should not require recalibration under normal circumstances.

There are two recommended approaches to verifying spectral calibration.

Option 1: A measurement of a scattering solution

Prepare a solution of LUDOX® or Glycogen and insert it into the FL-3D instrument's sample holder.

Set up a Scan acquisition using Nanometers as the CCD units across the full area of the chip. Use a resolution of 1 pixel by 1 pixel. Set the slits to 0.10mm, and the exposure time to 1 sec. initially. After taking an initial acquisition, reset the exposure time to yield between 20,000 and 60,000 cts at the highest peak on the scan.

The scan should look like the one below. Check along the first order Rayleigh scatter band to verify that the excitation $\lambda =$ emission $\lambda \pm 5$nm. If this is acceptable then the instrument is properly calibrated. If you find any problems with the calibration, please recheck your scan parameters to make sure they were set properly and repeat the acquisition. If the calibration is still off, then please contact the Fluorescence Service Department. It is helpful to have a copy of your data available to send to Instruments S.A. when you contact Fluorescence Service.
Option 2: A measurement of a standard Fluorophore

You may choose to collect the EEM of a standard Fluorophore to verify the instrument calibration in your specific region of interest. An example of a standard to use is Fluorescein, which excites at about 488nm, and emits at 513nm. Regardless of which standard you use please verify that you have factored in its sensitivity to temperature, pH, concentration and solvent. Prepare an acquisition to collect a scan of the EEM using nanometers for the CCD Units. Set the slits and exposure time to give a peak signal intensity of 20,000 – 60,000 counts.

Verify that the peaks occur at the proper wavelengths to verify the spectral calibration. If they do then the calibration is verified. Otherwise, please recheck your scan parameters to make sure they were set properly and repeat the acquisition. If the calibration is still off, then please contact the Fluorescence Service Department. It is helpful to have a copy of your data available to send to Instruments S.A. when you contact Fluorescence Service.

The Spex 3D spectrofluorometer will provide years of reliable service with minimal maintenance. Monitoring the lamp usage and replacing it at regular intervals is highly recommended. Additionally, monthly checks should be made to verify that the instrument calibration is still accurate. These checks may be made using a scattering sample or a standard fluorophore. The next chapter, Troubleshooting contains information for solving problems outside of normal maintenance, and also instructs on how to obtain help from the Fluorescence Service Department.
CHAPTER X

Troubleshooting

Your Spex 3D spectrofluorometer system has been designed for years of reliable use. In the event that a problem does occur, please refer to this chapter for help in diagnosing and troubleshooting the problem. If you need assistance with resolving a problem with your spectrofluorometer, this chapter also provides information on how to contact Fluorescence Service for assistance.

See if the problem is listed in the table below. If it is, try the suggested solutions. Be sure to carefully note the steps you have taken to remedy the problem and the result. Refer to the appropriate section of this manual and the software manuals if necessary.

Service Policy

If you find that you require technical assistance in resolving a problem with your FL-3D spectrofluorometer you may contact Instruments S.A./SPEX Fluorescence Group directly at (732)-494-8660 X160 in the United States, or contact your local representative worldwide. Additionally, you may contact us via our website at WWW.ISAINC.COM/FLUOR.

In order to minimize instrument downtime, our Service Engineers will make every effort to help correct, reduce or localize the problem through discussion with you. In order to help facilitate this please have the instrument’s purchase date, serial number, and system configuration available and be prepared to supply the software version as well. You will be asked to describe the malfunction and your attempts, if any, to correct it. Also, if you have provided any equipment to be used with the instrumentation (e.g. host computer), please have information on this equipment available as well.

The Spex-3D instrument is covered by a warranty that is printed on the back inside cover of this manual. Technical service is available for instruments that are out of warranty, for a fee. Please contact Fluorescence Service or your local representative for instrument service information and estimates.

Return Authorization

A Return Authorization (RA) Number issued by Fluorescence Service must accompany all instruments, components and accessories returned to the factory for any reason. If in
the United States, you may contact the Fluorescence Service Department directly by phone at (732)-494-8660 X160, or by fax at (732)-549-5157. For locations outside the United States, please contact your local representative.
## Troubleshooting Table

<table>
<thead>
<tr>
<th>Problem</th>
<th>Possible Cause</th>
<th>Remedy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Problems installing Grams/32 Software.</td>
<td>Conflicts with active Windows drivers.</td>
<td>Restart the Computer in Windows SAFE MODE. Then try to install the software again.</td>
</tr>
<tr>
<td></td>
<td>Inappropriate Host Computer</td>
<td>Refer to Chapter 11: Technical Specifications for minimum host computer requirements.</td>
</tr>
<tr>
<td>Cannot Communicate with Spex 3D</td>
<td>No power to Spex 3D</td>
<td>Check Rocker Switch is in ON (1) position.</td>
</tr>
<tr>
<td></td>
<td>Check Line Cord is connected to proper AC voltage source.</td>
<td>Check Fuse on rear panel of Spex 3D is not blown.</td>
</tr>
<tr>
<td></td>
<td>No Power to CCD-3000</td>
<td>Check Rocker Switch is in ON (1) position.</td>
</tr>
<tr>
<td></td>
<td>Check Line Cord is connected to proper AC voltage source.</td>
<td>Check Fuse on rear panel of Spex 3D is not blown.</td>
</tr>
<tr>
<td></td>
<td>IEEE-488 cable improperly connected and/or configured.</td>
<td>Check that the correct communications settings are being used (refer to Appendix C GPIB Installation Notes).</td>
</tr>
<tr>
<td></td>
<td>Check that correct National Instruments Card is used.</td>
<td>Check that cable is properly connected.</td>
</tr>
<tr>
<td></td>
<td>Cables Misconnected</td>
<td>Check that the system is properly cabled. Refer to Chapter II for installation instructions.</td>
</tr>
<tr>
<td></td>
<td>Improper Software Configuration</td>
<td>Check that software has been installed properly. Verify that no new software packages or accessories are interfering with the Datamax software.</td>
</tr>
<tr>
<td></td>
<td>Verify proper layout is being loaded.</td>
<td></td>
</tr>
<tr>
<td>Light is not reaching the sample.</td>
<td>Lamp is not turned on</td>
<td>Power cycle instrument</td>
</tr>
<tr>
<td></td>
<td>Slits are not inserted properly</td>
<td>Check that slits are inserted fully into holders.</td>
</tr>
<tr>
<td>Signal intensity is low.</td>
<td>Improper Software Settings</td>
<td>Check Exposure Time, Number of Accumulations and Binning (resolution).</td>
</tr>
<tr>
<td></td>
<td>Wrong Slits Used</td>
<td>Use wider slits for excitation and emission spectrographs</td>
</tr>
<tr>
<td></td>
<td>Emission Shutter doesn’t open.</td>
<td>Recheck cable connections to CCD-3000 controller.</td>
</tr>
<tr>
<td></td>
<td>Lamp power supply not set to the proper current rating.</td>
<td>Contact the SPEX Fluorescence Service Department.</td>
</tr>
<tr>
<td></td>
<td>Lamp is too old</td>
<td>[\text{Lamp lifetime: 400 hours}]</td>
</tr>
<tr>
<td></td>
<td>[\text{Replace lamp.}]</td>
<td></td>
</tr>
<tr>
<td>No change in signal intensity (flat, featureless spectra)</td>
<td>No sample.</td>
<td>Sample must be set up properly in sample mount.</td>
</tr>
<tr>
<td></td>
<td>CCD Detector is Saturated</td>
<td>Reduce Slit widths and/or exposure time.</td>
</tr>
<tr>
<td></td>
<td>Individual pixels saturate at 65,535 count.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Optical density effects and self-absorption</td>
<td>Sample is too concentrated. Dilute sample by a factor of 10 or 100 and rerun experiment.</td>
</tr>
<tr>
<td>Erratic signal intensity.</td>
<td>Lamp unstable.</td>
<td>Allow 20 minutes for lamp to warm up before use.</td>
</tr>
<tr>
<td>Raman band superimposed on fluorescence scan.</td>
<td>Aqueous solutions and solvents have Raman bands which are at constant energy from the exciting wavelength.</td>
<td></td>
</tr>
<tr>
<td>Large peak at double the excitation wavelength.</td>
<td>Second-order effects due to the spectrograph.</td>
<td>Use cut-on filters to eliminate second-order peaks from interfering with your spectra.</td>
</tr>
</tbody>
</table>

*Table X-1: Troubleshooting Table for the FL-3D Spectrofluorometer*
This section is provided to facilitate and guide troubleshooting efforts. Follow the suggestions given in the troubleshooting table prior to contacting the Fluorescence Service Department. It is important to be familiar with the system's software as well as its hardware. Please try to investigate any possibility of the problem being caused by user error prior to scheduling a service visit.
CHAPTER XI

Technical Specifications

The FL-3D spectrofluorometer may be purchased with a computer system from ISA or the computer can be purchased from another source and the Spex 3D interfaced via an IEEE-488 communications link to the host PC. The following information provides detailed information regarding the specifications of the FL-3D spectrofluorometer and recommendations for host computer specifications.

Specifications are subject to change without notice.

**Spex 3D (FL-3D) Spectrofluorometer**

The specifications listed are for the standard FL-3D configuration.

**SOURCE**

75W Ozone free Xenon short arc.
Broadband spectral output from 250nm to over 1.0µm.
Rated for 400 hours average lifetime

75 – 300 Watt Power Supply

**SPECTROGRAPHS**

Custom Designed for FL-3D System
All reflective optics used to reduce chromatic and other spherical aberrations.

**Excitation**

133gr/mm Concave Holographic Grating
Coverage from 250-550nm

**Emission**

133gr/mm Concave Holographic Grating
Coverage from 250-750nm

**Slits**

2 sets of 3 sizes; 0.05, 0.1, & 0.25mm fixed marked for excitation (X) or emission (M)

**SAMPLE MOUNT**

8mm vertical height of cell required for measurement.
Dimensions: 5” X 5” X 7.5” (l,w,h)
### Technical Specifications

**MULTICHANNEL DETECTOR**

MTECCD-512X512-9 with CCD-3000

½” X ½” backthinned chip with UV2 overcoat.

#### Chip Active Area

12.3 X 12.3 mm

#### Pixel Size

24 X 24 µm

#### ADC Precision

16 Bit

#### Dynamic Range

50,000 – 1

#### Electrons/count

1 to 16 electrons in autogain

#### Dark Current

< 5 electrons/pixel/second

#### CCD Quantum Efficiency

Greater than 40% from 250-750nm

Up to 60% at 600nm

#### Readout Noise

10 to 20 e⁻ RMS per pixel or binned datapoint

#### Readout Rate

20 kHz min.

#### Operating Temperature

Thermoelectrically cooled

(temperature not specified)

#### Exposure Time

0.010 to 5 seconds

#### Binning (Pixel Resolution)

1 pixel to 1/3 of usable chip in X or Y directions.

#### Usable Pixel Range

(due to optical constraints on excitation.)

<table>
<thead>
<tr>
<th>Excitation</th>
<th>Emission</th>
</tr>
</thead>
<tbody>
<tr>
<td>100-412</td>
<td>1-512</td>
</tr>
</tbody>
</table>

#### MCD Shutter

Uniblitz Shutter Located in Emission Spectrograph

Cycle Time below 1 msec
FULL INSTRUMENT SPECIFICATIONS

Sensitivity
50 picomolar Fluorescein

Wavelength Accuracy
+/- 5nm

Resolution
Better than 10nm Excitation
Better than 10nm Emission

Acquisition Time
300 X 500nm EEM acquisition in less than one (1) second.

Dimensions (l x w x h)

FL-3D
21.5" X 21.5" X 12.5" (with cables connected)

CCD-3000
11.5" X 13" X 6" (with cables connected)

Weight

Total
80 lbs (65 kg) (Not including host computer)

FL-3D
55 lbs

CCD-3000
25 lbs

Ambient temperature range
15-30 C

Relative humidity maximum
75%

Power requirements:

FL-3D
5 amps at 120V
2.5 amps at 240V
50/60 Hz single phase

CCD-3000
2 amps at 120V
1 amp at 240V
50/60 Hz single phase
**Host Computer**

Although ISA offers state-of-the-art computer systems and loads all software prior to shipment, you have the option of providing your own computer and installing the software. It is important to make sure that the computer which will be used meets the following minimum requirements.

<table>
<thead>
<tr>
<th><strong>Microprocessor/Clock Speed</strong></th>
<th>Pentium(586) min. recommended.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Operating System and Environment</strong></td>
<td>Windows 95™</td>
</tr>
<tr>
<td><strong>Floppy Drive</strong></td>
<td>A 3 ½-inch floppy disk drive.</td>
</tr>
<tr>
<td><strong>Fixed Disk</strong></td>
<td>A hard disk with at least 40 megabytes of free storage.</td>
</tr>
<tr>
<td><strong>Memory</strong></td>
<td>16MB RAM.</td>
</tr>
<tr>
<td><strong>Graphics Adapter</strong></td>
<td>SVGA Monitor and interface card.</td>
</tr>
<tr>
<td><strong>Keyboard</strong></td>
<td>A 10- or 12-function keyboard.</td>
</tr>
<tr>
<td><strong>Available Ports</strong></td>
<td>One parallel port for a dot-matrix printer One serial port for optional plotter or mouse</td>
</tr>
<tr>
<td><strong>GPIB Communication</strong></td>
<td>Host Computer must have one free slot ISA, PCMCIA, or PCI slot for compatible National Instruments GPIB IEEE-488 Interface Card.</td>
</tr>
</tbody>
</table>
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APPENDIX B

Glossary

This appendix contains a glossary of reference terms for fluorescence spectroscopy, total luminescence spectroscopy and other related terms. Please review these terms as needed for their specific meanings in the context of use with the FL-3D spectrofluorometer instrumentation.

Absorption:

The electronic transition from the ground state to the excited singlet state by absorbing a photon of light of a particular energy. This process typically occurs on time scales of $10^{-15}$ seconds.

Absorbance:

A quantity defining the extent of absorption by a substance. Absorbance is expressed as $-\log T$ where $T$ is the transmittance of the sample. Absorbance is also synonymous with optical density (od) where

$$OD = \epsilon(\lambda)cx$$

$\epsilon(\lambda) =$ the extinction coefficient (M$^{-1}$ cm$^{-1}$)

$c =$ sample concentration (M)

$x =$ path length (cm)

Accumulation:

An individual CCD exposure to the sample signal for a data collection. Multiple accumulations are summed together and are used to optimize the signal-to-noise of a measurement.

ADC:

An Analog to Digital Converter (ADC) converts a sample of an analog voltage or current signal to a digital value. The value may then be communicated, stored, and manipulated mathematically. The value of each conversion is generally referred to as a datapoint.

Air Cooled TE CCD

A compact two-stage charge coupled device that is thermoelectrically cooled using forced air for heat exchange. The device is cooled to below 250K (-23°C), providing a dark signal level of about 3,000-5,000 counts.

See also: Charge Coupled Device (CCD)

Backthinning:

The depletion layer of a CCD (where the photoelectric effect occurs) is normally partially obscured under the electrode gates, which are formed in layers above the depletion layer. This is due to the constraints of the chip fabrication process. The substrate (or back) of a CCD chip can
be etched down to be very thin. Then the chip is mounted so that the signal light is incident on the back rather than the front. The chance a photon has of reaching the depletion region is greater. Thus, the Quantum Efficiency is higher.

**Bandpass:**
The spread of light passing through the excitation and emission spectrometers. The wider the bandpass the higher the signal intensity, but the lower the resolution.

**Bandpass Filter:**
An optical element which selectively transmits a narrow wavelength range of light.

**Binning:**
The charges of adjacent pixels can be combined in the readout register cell for that column or row. This combining is called binning, or resolution for the FL-3D instrument.

The signal level increase is directly proportional to the number of pixels binned. However, shot noise only increases as the square root of the number of pixels (Felgett's S/N Advantage). Thus, signal to noise ratio is improved. Readout- associated noise is also reduced because the total signal from the binned number of pixels is combined into a single analog to digital conversion.

**Bioluminescence:**
Emission of light originating from a chemical reaction taking place in a living organism.

**Blaze wavelength (of gratings):**
Wavelength at which a grating is optimized for efficiency. Generally, the gratings are usable from 2/3 of the blaze wavelength to twice the blaze wavelength. The excitation and emission gratings for the FL-3D are blazed for efficiency in the UV and visible.

**Charge Coupled Device (CCD):**
The CCD is a solid state photodetector array made of silicon. It is essentially one continuous photosensitive material. Individual pixels or picture elements are defined by a grid of three electrode gates in the X and Y directions. The charge is collected under the gate with the greatest potential. During the readout cycle, the voltages applied to the gate electrodes are manipulated to move the charge across the pixels to the output register at the edge of the array. In contrast, PMT’s and other single channel detectors can measure only one intensity at a time. Photodiode Array detectors measure both intensity and wavelength. The CCD simultaneously measures intensity, wavelength and position differences projected along the height of the spectrograph image plane.
Charge Transfer Efficiency (CTE):
The percentage of charge moved from one pixel to the next is the charge transfer efficiency. The CCD has a high CTE if the pixels are read out slowly. As the speed at which the charge is transferred is increased, increasing amounts of the charge is left behind. The residual charge combines with the charge of the next pixel as it is moved into the cell. Therefore, using too high a transfer rate deforms the image shape; it smears the charge over the pixels that follow in the readout cycle. Temperature also affects CTE. Below -140°C the movement of the charges becomes sluggish, and, again, the image becomes smeared.

Chemiluminescence:
Emission of light originating from a chemical reaction.

Cosmic Ray Events:
Cosmic Rays are high energy particles from the sun. Although they penetrate all detectors, their effect usually is masked by dark current. The dark signal of a cooled CCD is low enough that cosmic rays may be detected. Variable Gain can help to reveal weak signals. Mathematical treatment of the data can also be used to remove the spurious spikes in spectra. Refer to the software manual for more about cosmic ray spike removal.

Cut-on filter:
Optical component which passes light of a higher wavelength.

Cut-off filter:
Optical component which passes light of a lower wavelength.

Dark Signal:
Dark signal is generated by thermal agitation. This signal is directly related to exposure time and increases with temperature. The dark signal doubles with every 7°C increase in chip temperature above -25°C. The more dark signal, the less dynamic range for experimental signal. This signal accumulates for the entire time between readouts or flushes, regardless of whether the shutter is open or closed. Dark signal is also generated during the charge transfer cycles of the CCD.

Data Extraction
The process by which an individual spectra can be selectively removed and saved as a separate datafile. Grams/32c supports the extraction of excitation, emission and synchronous spectra from the multifiles collected by the FL-3D.
Dynamic Range:
The **Dynamic Range** is the ratio of the maximum and minimum signal measurable. The dynamic range of the chip can be greater than that of the system which is limited by the **ADC**. A 16 bit ADC limit is $65,535 \left(2^{16} - 1\right)$ counts. A 14 bit ADC is limited to 16,383 counts. **Variable Gain** can be used to shift the ADC range to match the potential well capacity or signal levels of a given spectral measurement. In this way, stronger or weaker signals can be accommodated with optimal Dynamic Range.

On a pixel by pixel basis, the most intense detectable signal, the saturation level, is the lesser of the **Potential Well Capacity** of the pixel or the ADC maximum limit. When pixels are binned, individual pixels within a binned area may saturate if the intensity is concentrated. Also, the well capacity of the readout register will limit the total signal that can be binned from a given row or column of a binned area.

The weakest detectable signal is limited by the **Dark Level** plus the **Readout Noise**.

Electrons/Count:
Electrons per count is a value indicating how many electrons are needed to be identified by the **ADC** as the smallest measurable unit, or Count. The total “Counts” of a given datapoint are comprised of electrons from a variety of sources, including: Photoelectrons (signal), Dark Level, Read Out Noise, Bias Level, and Amplifier Noise.

Emission scan:
Shows the spectral distribution of light emitted by the sample. These scans are extracted from the excitation/emission matrix at a fixed excitation wavelength.

Energy transfer:
The transfer of the excited energy from a donor to an acceptor. The transfer occurs without the appearance of a photon and is primarily a result of dipole-dipole interactions between the donor and acceptor.

Excitation/Emission Matrix (EEM)
A three-dimensional plot showing the total luminescence from a sample across all useful wavelengths. The FL-3D instrument can acquire EEM’s with excitation from 250-550nm and emission from 250-750nm. Total luminescence spectroscopy is devoted to measurements of these EEM’s for various materials. See also: **Multifile, Total Luminescence Spectroscopy**

Excitation scan:
Shows the spectral distribution of light absorbed by the sample. These scans are extracted from the excitation/emission matrix at a fixed emission wavelength.

Extrinsic fluorescence:
Inherent fluorescence of probes used to study non-fluorescent molecules.

Fast Kinetics Measurements
Fluorescence studies which require rapid data collection to resolve sample reactions, environmental changes and other events which can affect a samples properties. The FL-3D’s
rapid collection of EEM’s may be employed to fully observe the effects of these rapid events on the total luminescence of a sample.

**Felgett's Advantage:**
Multichannel detection provides an improvement in signal to noise ratio, as compared to single channel (scanned) spectral detection. Because the multichannel detection acquires a number of spectral elements simultaneously, the S/N is improved by a factor proportional to the square root of the number of channels acquired.

**Fluorescence:**
The emission of light or other electromagnetic radiation during the transition of electrons from the excited singlet state to the ground state. Fluorescence typically occurs on the time scale of $10^{-9}$ seconds.

**Fluorescence lifetime:**
The average length of time that a molecule remains in the excited state before returning to the ground state.

**Flush:**
To reduce noise and maximize dynamic range at the CCD, the dark charge that has accumulated on the chip can be rapidly removed by flushing. The effect of flushing the array is similar to a readout cycle in that the charges are cleared from the pixels. But a flush dumps the charges without conversion. A flush is much faster than a readout. Flushing is only necessary when there is an appreciable time between readouts.

**Frequency Domain lifetime method:**
A technique for measuring fluorescence lifetimes where a sample is excited with light whose intensity is modulated sinusoidally. The emission is a forced response to the excitation, that can be easily detected and recorded. Fluorescence lifetimes are calculated from both the phase angle difference between the sample and a standard, and the demodulation factor for both samples.

**Front face detection:**
A mode of detection in which fluorescence is collected off the front surface of the sample. Front face detection is usually selected for samples such as powders, thin films, pellets, cells on a coverslip and solids.

**Full Well Capacity:**
**Full well capacity** is the measure of how much charge can be stored in an individual pixel. This specification varies for each chip type. It depends on the doping of the silicon, architecture and pixel size. The quantum well capacity is usually around 300,000 electrons. The greater the well, the greater the **Dynamic Range**. A chip with a larger full well capacity can record a higher signal level before saturating. See also **Variable Gain**.

**Grating:**
An optical element in a spectrometer or spectrograph that uses finely etched vertical grooves to disperse incident light into its individual wavelengths.
IEEE-488 (GPIB) Interface
A standardized method of high speed data transfer that is employed by the CCD-3000 controller to transfer data to the host computer. A compatible interface card must be properly installed the host computer for use with the FL-3D instrument.

Intrinsic fluorescence:
The natural fluorescent properties of molecules.

Jacquinot’s Advantage:
Multichannel detection provides a throughput advantage over single channel detection in that no emission slit is required and, thus, the detector elements themselves are the limiting aperture for the spectrograph.

Laser:
A monochromatic light source which provides high excitation intensity.

Linearity:
When photo response is linear, if the light intensity doubles, the detected signal will double in magnitude as well. Nonlinear response at medium to high intensities is usually due to amplifier problems, and at very low light levels poor charge transfer efficiency. A CCD’s response is linear, once the bias is subtracted. Another definition of linearity is applied to the spectral positioning accuracy or tracking error of a spectrometer drive mechanism, see Spectral Calibration.

MCD Shutter:
Actually meaning, multi-channel device shutter, this Uniblitz shutter is selected for its rapid cycle time. This is a necessity when working with an extremely sensitive CCD detector.

Mercury lamp:
A light source which offers discrete narrow lines as opposed to a broadband source (e.g. Xenon). Mercury lamps are often used to verify the accuracy and resolution of a spectrometer or spectrograph.

Mirror Image Rule:
Usually, a sample which absorbs light for certain energy level transitions will exhibit an emission profile that appears to be the mirror image of the absorption spectrum. The reason for this is that the same energy level transitions are used for excitation and emission, with the transitions returning to the ground state being the complement to the those to the excited state.

Multifile:
The three-dimensional acquisition datafiles collected by the software and stored with an .SPC extension. These are the excitation-emission matrices collected by the software. For the FL-3D the X, Y, and Z axes represent the Emission $\lambda$, Excitation $\lambda$, and Signal Intensity, respectively. Multifiles may be worked with in their entirety, or the may be split up into individual two dimension spectra using emission, excitation or synchronous extractions.
Noise:

Noise is common to all detectors. The total amount of signal that exists is less important than the ratio of signal magnitude to noise magnitude (S/N). With a high signal to noise ratio a signal peak can be discerned even though signal counts may be low. The noise components for CCD arrays are as follows:

Amplifier Noise: Some noise is introduced in the process of electronically amplifying and conditioning the signal read from the detector before conversion to a digital value. Part of Readout Noise.

Conversion Noise: During the conversion of an analog signal to a digital datapoint some electronic noise is introduced, statistical variations occur in the least significant bit of the converted data. Part of Readout Noise.

Dark Noise: The detector will integrate a thermally generated Dark Current at all times, whether light is reaching the detector or not. Most of the dark current signal is a steady state level that can be subtracted, and so will not ultimately contribute to the noise. However, a component of Dark Current is Dark Noise due to statistical variations in the Dark Current. The Dark Noise component increases as the square root of the Dark Current. Dark Current, and therefore Dark Noise, can be reduced by cooling. For this reason the CCD for the FL-3D should be cooled to below 250K (-23°C). If the signal level is below saturation, increasing the signal integration time per readout will minimize the effect that dark noise has on the acquired signal. If the signal level is too high, summing multiple reads can give similar improvement. (See Readout Noise below.)

Readout Noise: The electronic noise impressed on the signal during the readout and digitizing of the signal. For convenience, usually all of the noise associated with resetting, amplifying, and converting the signal are considered as readout noise. When averaging signal by acquiring over a long interval of time, increasing the signal integration time per readout rather than summing multiple readouts is preferred. This will proportionately reduce the readout noise component in the acquired signal. However, the integration time must be short enough to prevent saturation of any individual pixels and to keep the digital signal for any datapoint below the ADC limit.

Reset Noise: Following pixel or bin readout, the readout register is reset to a level approaching zero charge. Reset Noise is the non-uniformity in the resetting. This is canceled by Correlated Double Sampling. Part of Readout Noise.

Shot Noise: This is due to the random statistical variations of light. It includes both experimental and dark signal components. Shot noise is equal to the square root of the number of electrons generated. Its effect can be minimized by increasing signal intensity, signal integration time, or summing a number of readouts.

Optical density effects:

Fluorescence intensities are proportional to the concentration over a limited range of optical densities. High optical densities can distort the emission spectra as well as the apparent intensities.

Phosphorescence:

The emission of light or other electromagnetic radiation during the transition of electrons from the triplet state to the ground state. Phosphorescence is generally red-shifted relative to fluorescence and occurs on time scales ranging from $10^{-6}$ to several seconds. To enhance phosphorescence detection, samples are often frozen at liquid nitrogen temperature.
Photoelectric Effect:
Some materials respond to illumination from photons by releasing electrons. When light of sufficient energy hits a photosensitive material, an electron is freed from being bound to a specific atom. Such materials include the P-N junctions of the silicon photodiodes used in CCD arrays. The energy of the light must be greater than or equal to the binding energy of the electron to free an electron. The shorter the wavelength, the higher the energy the light has.

Photoelectron
A photoelectron is an electron that is released through the interaction of a photon with the active element of a detector. The photoelectron could be released either from a junction to the conduction band of a solid state detector, or from the photocathode to the vacuum in a PMT. A photoelectron is indistinguishable from other electrons in any electrical circuit.

Photo Response NonUniformity (PRNU)
PRNU is the peak to peak difference in response between the most and least sensitive elements of an array detector, under a uniform exposure giving an output level of \( V_{\text{Sat}}/2 \). These differences are primarily caused by variations in doping and silicon thickness.

Pixel
An individual detector element on a CCD chip. Each pixel acts an individual silicon photodiode with its sensitivity dependent on the coating and substrate thickness used. Pixels on the CCD detector used by for the FL-3D measure 24uM x 24uM and have an UV overcoat on a backthinned chip.

Pulse sampling lifetime method:
A technique for measuring fluorescence lifetimes where an initial population of fluorophores are excited by infinitely short pulses of light. An advantage of this technique is the direct recording of time-resolved emission spectra.

Quantum Efficiency (QE):
The efficiency of the photoelectric effect of a detector can be quantified. The quantum efficiency of a detector is the ratio of number of photoelectrons produced to the number of photons impinging on a photoactive surface. A QE of 20% would indicate that one photon in five would produce a distinguishable photoelectron. CCD’s are made of silicon which has a high QE, about 45-50% at its peak at 750 nm. The quantum efficiency of a detector is determined by several factors. These include the material's intrinsic electron binding energy or band gap, the reflectivity of the surface, the thickness of the surface, and energy of the impinging photon (h \( \nu \)). The QE varies with the wavelength of incident light. Standard CCD’s typically have a peak QE of about 50%. Back thinned CCD’s may peak at about 85%. The QE at short wavelengths can be improved by coating with fluorescent dye that converts UV light to longer wavelengths where the quantum efficiency of the chip is higher.

Raman scattering:
Scattering caused by vibrational and rotational transitions. Raman bands generally appear red-shifted relative to the incident electromagnetic radiation. The primary characteristic of Raman scatter is that the difference in energy between the Raman peak and the incident radiation is constant in energy units (cm\(^{-1}\))
Rayleigh scattering:
Light scattering from such particles as atoms or small molecules whose dimensions are much smaller than the wavelength of incident light. Scattered light is of the same energy as the incident light. The characteristics of Rayleigh scatter is that the scatter radiation intensity is inversely proportional to the 4th power of the wavelength of incident radiation.

Rayleigh-Tyndall scattering:
A combination of Rayleigh and Tyndall scatter. These two scattering phenomena cannot be separated so that if the molecule's Stokes shift is small, Rayleigh-Tyndall scatter will limit the ultimate resolution.

Readout Time:
The Readout Time of a CCD is the interval required to move the charges from their locations in the array to the readout registers, sample the charges, amplify them and convert them to datapoints. A consideration with a CCD is that the time between sample exposures can be longer than linear array detectors. This is because the readout requires that the charges be moved across the array (charge coupled). Also, the correlated double sampling readout technique requires more time per pixel.

Resolution:
(1) The software setting which sets the binning of adjacent pixels used for a measurement on the FL-3D instrument. (2) The ability of a spectrometer or spectrograph to separate two closely spaced peaks. Resolution can be improved by decreasing the number of pixels binned together or the slit widths in the instrument.

Responsivity
Responsivity is the ratio of output voltage to corresponding exposure (µJ/cm²). Technically it is measured at \( V_{Sat\ min}/2 \) under specified conditions of illumination, readout rate, and temperature.

Right angle detection:
Collection of fluorescence at 90 degrees to the incident radiation. Right angle detection is typically selected for dilute and clear solutions.

Saturation Level
The maximum signal level that can be accommodated by a device is its saturation level. At this point, further increase in input signal do not result in a corresponding increase in output. This term is often used to describe the upper limit of a detector element, an amplifier, or an ADC.

Scatter:
A combination of Raman, Rayleigh and Rayleigh-Tyndall scattering that can distort fluorescence spectra with respect to intensities and wavelengths.

Singlet state:
The spin paired ground or excited state. The process of absorption generally produces the first excited singlet state which gives time to fluorescence and can undergo intersystem crossing to form a triplet state.
Glossary

**Spectral Calibration:**
The process by which the area of the CCD chip is accurately converted to the actual wavelengths of the excitation-emission matrix incident upon it. *Appendix D* reviews the software procedures used to perform spectral calibration for the instrument.

**Spectral Response:**
Most detectors will respond with higher sensitivity to some wavelengths than to others. The spectral response of a detector is often expressed graphically in a plot of responsivity versus wavelength.

**Spectrograph**
A component in a fluorometer system that contains a dispersive optic or set of optics with a multichannel detector such as a CCD on its exit port. Some of these devices are scanned, while others simply collect the full spectra in one collection by the multichannel detector. The FL-3D uses a fixed grating *spectrograph* to collect the entire EEM in one collection of the CCD.

**Spectrometer:**
A component in a fluorometer system which is scanned to provide the excitation and emission spectra. Spectrometers have an exit slit and use single channel devices such as photomultiplier tubes or silicon photodiodes to collect spectral data. Spectrometers are selected for performance related to low stray light, resolution and throughput.

**Stokes shift:**
Generally, it is quantified as the energy difference between the absorption peak of lowest energy and the fluorescence peak of maximum energy.

**Synchronous scan:**
Spectra which characterizes the overlap between the excitation and emission. This is observed and extracted as a diagonal along the excitation/emission matrix with a constant offset.

**Total Luminescence Spectroscopy (TLS)**
Spectroscopy devoted to monitoring changes to the entire excitation/emission matrix of luminescence on a sample. This discipline of spectroscopy is most readily applied to fast kinetics measurements of samples during reactions, temperature curves or changes in other variables.

**Triplet state (T1):**
The spin paired ground or excited state that is formed from the excited singlet state when paired electrons become unpaired. The triplet state gives rise to phosphorescence.

**Tyndall scattering:**
Scatter which occurs from small particles in colloidal suspensions.

**UV Overcoating (Enhancement):**
Although silicon junctions release photoelectrons when illuminated with UV light, the depth of penetration into the silicon is very shallow. With this shallow penetration, the probability of a UV photon penetrating to the depletion zone is less than for longer wavelength photons. Thus the QE is lower in the UV than in the visible and NIR. By coating the chip with a fluorescent dye that converts UV light to longer wavelengths, the probability of photon detection is increased. This is
because the longer wavelength photons emitted by the excited dye are able to penetrate to the depletion layer of the CCD.

**Variable Gain:**

If a CCD has a quantum efficiency of 50%, then with two incident photons, a chip would produce one electron. With an electronic signal gain that produces one ADC count per electron, the system can measure that single electron, or two photons. If one ADC count equates to four electrons, then eight photons would be needed to produce those four measurable electrons, and thus one count. With a higher gain, one e-/count, the ability to resolve weak signals close to the noise level is increased. This is extremely important in applications such as Raman, where signals are typically very weak. By increasing the gain to measure signal levels which are very close to the noise, one can improve the signal to noise ratio while maintaining the same integration time. Or, one can achieve the same signal to noise ratio in less time.

A low gain has its applications as well. It is useful for preserving the full dynamic range of the CCD chip when measuring more intense signal levels. With a 16 bit ADC and one count equaling one electron, the ADC can only count a total of 65,000 electrons (with QE=50%, 130,000 photons). When the one ADC count equals four electrons, the 16 bit ADC counts to a maximum of 4 x 65,000 or about 250,000 electrons (500,000 photons) which is close to the full well capacity of the chip.

Variable Gain is needed to allow optimization between maximum sensitivity and full chip dynamic range. It allows trading off the ability to distinguish a small signal from noise in order to increase the highest signal level measurable, or vice versa.

**Xenon lamp:**

Lamp which produces a continuum of light from the ultraviolet to the near infrared for sample excitation. These lamps are classified as broad-band sources.
IEEE 488 Communication with a Computer

The CCD-3000 is specified to be compatible with only National Instruments GPIB IEEE 488 interface boards for use in Windows 95 and Windows 98 operating system environments. The installation, connection, testing and troubleshooting of these boards is outlined in this appendix.

Supported IEEE 488 Computer Interface Boards

Of National Instrument's GPIB offerings, we support the following cards and drivers for use in Windows 95 or Windows 98.

- AT-GPIB/TNT 488 Interface board: The driver supplied by National should be version 1.2 or later. This board comes in Plug-and-Play and standard formats, either is suitable for use with the CCD-3000.

- PCMCIA-GPIB: Should have National Instruments driver 1.2 or newer.

- PCI-GPIB: Fits most newer Pentiums and takes advantage of free PCI slots. Should use National Instruments driver 1.2 or newer.

- GPIB-PCII/PCIIA 488.2 Interface boards: The driver supplied by National should be version 1.2 or newer, and their BASIC support disk should be version 2.0 or newer. These boards should only be used in PCII mode, as the PCIIA driver can conflict with Windows 95.

There are other boards by National and other suppliers for IBM compatible and Macintosh computers. Many of these boards can function in a SIMILAR fashion, however the program communicates specifically with the National Instruments driver therefore we do not support or guarantee reliable communications with other boards and software, we strongly recommend that you use those National Instruments products described above.
Installing a Plug-and-Play GPIB Board in your Host Computer
(AT-GPIB/TNT, PCMCIA-GPIB, PCI-GPIB boards)

Please consult the National Instruments GPIB *Getting Started* manual for instructions on the proper installation of the GPIB board into your host computer. There must be adequate physical space for the board in the correct slot within the host computer. Also, the host computer must have adequate hard drive space for the GPIB drivers which must be installed.

With Plug-and-Play installation, these boards are fairly easy to install and set up, when you follow the National Instruments instructions. The *Base I/O Address, DMA Channel* and *Interrupt (IRQ) line* are configured by Windows during the installation. When the installation is complete you should reboot the host computer before testing communications with the board.

To get the latest available National Instrument drivers you may access the GPIB section of the National Instruments website at [WWW.NATIONALINSTRUMENTS.COM](http://WWW.NATIONALINSTRUMENTS.COM) for downloadable files.

The first example assumes that a Plug-and-play AT-GPIB/TNT board is being installed using national Instruments V1.30 software driver on Windows 98. The GPIB board names is GPIB0 and the used device name is DEV5.

**Example of Plug-and-Play GPIB Board configuration:**

The following screenshots display the *System Properties* within the *Windows 98® Control_Panel* for an AT-GPIB/TNT board installed on a Pentium II 350MHz PC. This configuration is meant as an example for reference only, as each PC and GPIB card may require different configurations for the DMA, Interrupt and I/O addresses.

![Figure C-1: Device Manager – GPIB](image1)
![Figure C-2: Device Manager GPIB General Settings](image2)

Selecting the GPIB card *Properties* in the *Device Manager* brings up the card configuration settings and driver settings. These settings define the capabilities and defaults for the GPIB card. The board settings should be set as shown in Figure C-3 & C-4.
Information on the type and version for the GPIB driver may be found in the **Driver** section of GPIB properties, shown in figure C-6. The specific resource settings are set and/or displayed in **Resources**, figure C-7.
Selecting the *National Instruments GPIB Interfaces* Properties in the Device Manager brings up the specific device configuration settings or Device Templates. These are shown in figure C-8.

![Figure C-8: GPIB Interface Device Templates](image)

### Installing the PCII/PCIIA GPIB Board in your Host Computer

The PCII/PCIIA GPIB card is a Windows 3.1 or Windows 95 compatible GPIB card that is *not* plug-and-play. Please consult the National Instruments GPIB *Getting Started* manual for instructions on the proper installation of the GPIB board into your host computer. There must be adequate physical space for the board in the correct slot within the host computer. Also, the host computer must have adequate hard drive space for the GPIB drivers which must be installed.

The *Base I/O Address*, *DMA Channel* and *Interrupt (IRQ) line* must be properly configured with jumper settings on the GPIB board prior to installing it in the host PC. The proper setting of these values depends on the specific configuration of the host computer. Many apparent software problems can be avoided by verifying that these settings are correct with no hardware conflicts on both on the GPIB board and in the Windows properties for the board.

The next example is for installation of a PCII/PCIIA card (as a PCII card) using National Instruments V1.2 software driver for Windows 95. The PCII board name is GPIB0 and the first device name as DEV5. Follow the directions sent with your software drivers for installation instructions. To get the latest available National Instrument drivers you may access the GPIB section of the National Instruments website at [WWW.NATIONALINSTRUMENTS.COM](http://WWW.NATIONALINSTRUMENTS.COM) for downloadable files.

**Examples of GPIB PCII/IIA configuration:**
The following screenshots display the **System Properties** within the **Windows 95® Control Panel** for a PCII/IIA installed on a Pentium 266MHz PC. These are meant as examples for reference only, as each PC and GPIB card may require different configurations for the DMA, Interrupt and I/O addresses.

Selecting the GPIB card **Properties** in the **Device Manager** brings up the card configuration settings and driver settings. These settings define the capabilities and defaults for the GPIB card. The NI-488.2 settings should be set as shown in Figure C-11 & C-12.
Information on the type and version for the GPIB driver may be found in the **Driver** section of GPIB properties, shown in figure C-13. The specific resource settings are set and/or displayed in **Resources**, figure C-14.
Selecting the *National Instruments GPIB Interfaces Properties* in the *Device Manager* brings up the specific device configuration settings or *Device Templates*. These are shown in figures C-15 & C-16.

![Figure C-15: Device Manger – National Instruments GPIB Interfaces](image1)

![Figure C-16: GPIB Interface Device Templates](image2)

**Verifying GPIB Communications**

Please also refer to the documentation provided with your computer's IEEE 488 interface for information about testing IEEE 488 communication and the various commands which may be used. You must already have the GPIB board installed, and appropriate software drivers installed before proceeding to this section.

Go to the *Start Menu* in Windows. Select the *NI-488.2 Software for Windows* program item. This menu is shown in Figure C-17.

Select the *Diagnostic* option to test your card configuration, a screenshot is displayed of this in Figure C-18.

![Figure C-17: Start Menu option for NI-488.2 Interface](image3)

![Figure C-18: NI-488.2 Interface Diagnostic Program](image4)
Win32 Interactive Control allows you to send commands directly to the GPIB interface, while GPIB Spy allows you to monitor and capture the GPIB calls which are made. To test communications you may use the following procedure to write and read a character from the CCD-3000 via the GPIB card using Win32 Interactive Control and monitoring this with GPIB Spy.

Activate GPIB Spy and start capture then enter Win32 Interactive Control and enter the following lines.

```
IBFIND GPIB0
IBFIND DEV5
IBWRT " 
IBRD 1
```

If you have successfully communicated, you should receive a B or an F. If you have an error message displayed, refer to the National Instruments documentation to interpret it.

GPIB Spy should display the capture of the above similar to figure C-19.

![GPIB Spy capture of NI-488.2 Interface Test Procedure](image)

*Figure C-19: GPIB Spy capture of NI-488.2 Interface Test Procedure*
Spectral Calibration Procedure for the Spex 3D Spectrofluorometer

This appendix goes through the software algorithm and procedure for performing spectral calibration of the FL-3D. Each new instrument is calibrated at the factory, and will not require recalibration to be executed by the user except under special circumstances, when directed by Fluorescence Service or other company representative. If you feel that your system requires recalibration, please contact Fluorescence Service or your local Instruments S.A., Inc. representative, before performing this procedure.

Theory for Spectral Calibration of the 3D Spectrofluorometer

Spectral calibration for the 3D Spectrofluorometer involves associating a particular pixel coordinate (X,Y) with the appropriate wavelength position (Excitation $\lambda_e$, Emission $\lambda_i$) for all usable pixels on the CCD detector. An excitation/emission matrix is distorted by the spherical aberrations created by the optics used to image the EEM as well as by magnification of the incident beams at the sample and at the CCD detector.

The spectral calibration procedure corrects both the excitation and emission axes through the use of two acquisitions. The first acquisition is of a spectral line source (low pressure Hg lamp). This is used to correct for any spherical aberrations which result in a distortion of the emission spectra upon the CCD detector. Additionally, a “smart” algorithm locates the spectral peak positions in pixels and finds their correct wavelength positions. This calibrates the emission path of the instrument.

A scattering sample (Ludox®) is scanned to find the first order Rayleigh (scatter) band across the excitation/emission axis. Another “smart” algorithm locates the first order Rayleigh peak pixel positions and assigns the proper wavelength position along the excitation axis. When complete the software generates coefficients for 2nd order polynomial expressions of the calibration factors for each axis. The coefficients are applied when the user selects nanometers for the CCD Units in normal use of the system.
Files Used for the Calibration Procedure

**PEAKTBL.AB**  
Macro Program used to split a multifile into a group of subfiles, then generate a peak report (.PKS) file for each subfile.

**MATCHING.EXE**  
Identifies mercury emission peaks on each peak report file specified. Outputs a datafile of the identified peak positions in pixels and corresponding mercury emission wavelengths (WAVEPIX.DAT). Also, creates a text file (REPORT.TXT) containing this information as well as a matching success rate.

**CALCABC.EXE**  
Calculates the emission calibration polynomial coefficients for every usable excitation pixel. This data is stored in a binary file (CCDEXP.BIN). A text version of the results are stored as ABC.TXT.

**CALIBEXT.EXE**  
Calculates the excitation calibration polynomial and stores the data in CCDEXP.INI. A text version of the results is saved in CALIBEXT.TXT.

**WAVELEN.TXT**  
Datafile containing the theoretical positions of the most prominent mercury emission lines.

Files Created or Modified by the Calibration Procedure

**CCDEXP.INI**  
Initialization file for the CCD. Excitation calibration polynomial coefficients are saved here. A representative emission calibration polynomial (for a pixel near the center of the chip) is saved here as well as a default for the display. The file also contains important information on the chip size and pixel spacing.

**CCDEXP.BIN**  
Binary file containing the data output from CALCABC.EXE. Contains emission calibration polynomial coefficients for each excitation pixel subfile matched properly.

**ABC.TXT**  
Text file showing the results of CALCABC.EXE. Shows data stored in CCDEXP.BIN.

**WAVEPIX.DAT**  
Datafile containing the results from MATCHING.EXE. Contains the pixel position of each found mercury emission
FL-3D with Datamax-3D  

Spectral Calibration Procedure for the FL-3D

as well as its corresponding theoretical wavelength position.

REPORT.TXT  
Text file showing the results from MATCHING.EXE which are saved in WAVEPIX.DAT. At the end of this file is a report of the matching rate for the data.

CALIBEXT.TXT  
Text file showing the results of CALIBEXT.EXE. Shows the first order Rayleigh peaks found and their corresponding wavelength positions (EXC. EM) for all subfiles. At the end of this file the excitation calibration polynomial is shown.

Spectral Calibration Procedure for the FL-3D

Equipment Required
Low Pressure Mercury Lamp
Ludox® Sample in 4ml Quartz Cuvette.  
  Combine 0.5ml 40 wt.% Solution of Ludox with 3.5ml of deionized water.
OD 1 Neutral Density Screen

Before Beginning the Procedure
Please back up the following files from your GRAMSN directory before starting the procedure. They contain the current spectral calibration coefficients for your instrument.

CCDEXP.INI
CCDEXP.BIN

IMPORTANT: For Grams/32c to properly execute peak picking for multifiles, the Display Settings in Windows 95 must be set for a non-accelerated 16 or 256 color screen driver. Accelerated drivers can cause errors in the peak picking routine.

1. Power up the FL-3D Instrument in the following order.
   1.1. FL-3D Instrument (rocker switch on rear)
   1.2. CCD-3000 Controller
   1.3. Host Computer, Monitor and Peripherals

2. Before running Datamax-3D, Copy CCDEXP.INI from the GRAMSN directory to the CCDCALIB subdirectory of GRAMSN.

3. Run Datamax-3D.

4.1. Remove the ¼” plastic plug on the outside wall of the left side of the sample compartment.

4.2. Block the Xenon lamp emission by inserting an entrance slit in its slot backwards.

4.3. Tape the OD 1 Neutral Density Screen over the hole on the inside of the instrument. Replace the Cover. Verify that there is no sample in the sample holder.

4.4. Set up the Low Pressure Mercury Lamp on the outside of the instrument. So that the bulb is directly facing the hole. Turn on the lamp.

Figure D-1: Inserting ND Screen in the FL-3D
Figure D-2: Setting up the Hg Lamp Scan
4.5. Define an Emission Scan for the Mercury Spectra as shown in the screenshot below. Save the file as HG.SPC. Run the Acquisition.

![Figure D-3: Hg Scan Parameters](image)

5. Calculate the Emission Axis Calibration.

5.1. Go to the View Menu and select Extract.VW. Verify that the highest peak intensity is between 20,000 and 65,000 counts. If not, adjust the exposure time and repeat the acquisition. Find the maximum and minimum excitation pixel positions for which you observe a mercury emission spectrum with at least four distinct peaks (>2,000 counts over background). Note these pixel positions and their corresponding subfile numbers.

5.2. Set up the Peak Marking parameters. Zoom in on the smallest mercury peak that you wish to detect and mark. Click Peaks/Auto Settings. This sets the thresholds and other peak parameters for peaks greater than the one specified. Then activateate Peaks/Mark All Peaks. The subfiles should now show peak markings.
5.3. Go to the Arithmetic Menu and select Do Program. Browse and select the macro program *Peaktbl.AB*.

5.3.1. Specify the slicing to start at minimum subfile and maximum subfile where usable spectra were found in step 5.1. Set the Slice Increment to be 1.

5.3.2. Click OK. *Peaktbl.AB* slices the multifile into individual subfiles for the specified range, then creates peak tables for each subfile. This data is used by the matching routine for locating the mercury spectral peaks in each subfile.

5.4. Minimize Datamax-3D.
5.5. Run *MATCHING.EXE* in the GRAMSN directory.

![Matching.EXE Opening Screen](image_url)

*Figure D-6: Matching.EXE Opening Screen*

5.5.1. Click Run.

5.5.2. Fill in the required information to run matching as shown below, except, use the minimum and maximum subfiles found in step 5.1.

![Matching.EXE Setup Options](image_url)

*Figure D-7: Matching.EXE Setup Options*

5.5.3. Click OK. The program should output that matching was completed successfully. If not, please return to step 4.

5.5.4. Click EXIT.

5.5.5. Using a text editor, open *REPORT.TXT* in the GRAMSN\CCDCALIB directory. At the end of this file a matching rate is reported. Verify that the matching rate is >95%, and that the first peak detected for all matched subfiles varies by only +/-10pixels (i.e. correct first peak is detected). Otherwise, please return to step 4.
5.6. **Run CALCABC.EXE in the GRAMSN directory.**

`CALCABC.EXE` calculates the excitation calibration polynomials for each subfile and stores them in `CCDEXP.BIN`. A text version of the results is created as `ABC.TXT`.

6. **Acquire the Ludox® EEM.**

6.1. **Turn OFF and remove the mercury lamp.**

6.2. **Place the Ludox sample in the sample mount.**

---

**Spectral Calibration Procedure for the FL-3D with Datamax-3D**

9 peaks read originally
Peak Table: c:\gramsn\data\\hgg290.pks

<table>
<thead>
<tr>
<th>No.</th>
<th>Wavelength</th>
<th>Pixels</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>253.600000</td>
<td>64.478005</td>
</tr>
<tr>
<td>1</td>
<td>312.600000</td>
<td>125.475340</td>
</tr>
<tr>
<td>2</td>
<td>365.400000</td>
<td>178.521680</td>
</tr>
<tr>
<td>3</td>
<td>404.600000</td>
<td>218.548630</td>
</tr>
<tr>
<td>4</td>
<td>435.800000</td>
<td>249.649050</td>
</tr>
<tr>
<td>5</td>
<td>507.200000</td>
<td>322.384220</td>
</tr>
<tr>
<td>6</td>
<td>546.000000</td>
<td>360.626070</td>
</tr>
<tr>
<td>7</td>
<td>576.900000</td>
<td>392.670010</td>
</tr>
<tr>
<td>8</td>
<td>625.100000</td>
<td>441.146230</td>
</tr>
</tbody>
</table>

Total Number of Peak Tables: 219
Total Number Matched: 219
Matching Rate: 100%

Figure D-8: End of Report.TXT showing Matcing.EXE Results

---

Figure D-9: Execution of CALCABC.EXE Routine
6.3. Restore Datamax-3D.

6.4. Define a Scan for the Ludox EEM as shown below. Set the filename as LUD.SPC. Run the Acquisition.

![Datamax-3D interface with scan parameters]

Figure D-10: Ludox Scan Setup

7. Calculate the Excitation Axis Calibration.

7.1. Go to the View Menu and select Extract.VW. Verify that the highest peak intensity is between 20,000 and 65,000 counts. If not, adjust the exposure time and repeat the acquisition. Find the maximum and minimum excitation pixel positions for which you observe a first order Rayleigh peak. Note these pixel positions and their corresponding subfile numbers.

7.2. Set up the Peak Marking parameters. Zoom in on the smallest mercury peak that you wish to detect and mark. Click Peaks/Auto Settings. Then activate Peaks/Mark All Peaks. The subfiles should now show peak markings.
7.3. Go to the Arithmetic Menu and select Do Program. Browse and select the macro program Peaktbl.AB.

7.3.1. Specify the slicing to start at minimum subfile and maximum subfile where usable spectra were found in step 7.1. Set the Slice Increment to be 10.

7.3.2. Click OK. Peaktbl.AB slices the multfile into individual subfiles for the specified range, then creates peak tables for each subfile. This data is used for the CALIBEXT.EXE routine.

7.4. Close Datamax-3D.

7.5. Run CALIBEXT.EXE in the GRAMSN directory. Specify the
slicing to start at minimum subfile and maximum subfile where usable spectra were found in step 7.1. Set the Increment to be 10.

![Excitation Calibration](image)

**Figure D-12:** Setup and Execution of CALIBEXT.EXE

7.6. **Click DISPLAY.** CALIBEXT.EXE calculates the coefficients for the excitation calibration polynomial and stores them in CCDEXP.INI.

7.7. **Click EXIT.**

8. Copy the calibration files to the GRAMSN directory.

8.1. Copy CCDEXP.BIN and CCDEXP.INI to the GRAMSN directory from the CCDCALIB subdirectory (overwrite the other files which were backed before starting the procedure).

9. Verify that the calibration has been done properly.

9.1. Restart Datamax-3D.

9.2. Repeat the Ludox EEM acquisition defined in step 6.4, except now set the CCD Units to *Nanometers*. Set the filename as CLUD.SPC.

9.3. Verify that the first order Rayleigh band is calibrated (i.e. the excitation wavelength = emission wavelength for first order Rayleigh). Also check the second order Rayleigh (emission wavelength = twice the excitation wavelength). Errors less than +/-5nm are expected. Repeat the entire procedure if you are not satisfied with the results.
Figure D-13: Ludox Scan in Nanometers after Spectral Calibration
This appendix contains printouts of the three main initialization files, CCD_LOC.INI, CCDEXP.INI and CCDLOAD.INI, for the Spex 3D spectrofluorometer within the Datamax software package. These printouts are included for reference purposes only and should not be modified unless directed to do so by a Fluorescence Customer Service Engineer.
CCD_LOC.INI

This file specifies the type of communication, controller device and Datamax virtual instrument information. The default controller is the CCD-3000 using IEEE-488 communication.

; SAQ.ini file which defines the structures and the number of ports available
; SERIAL=1
; IEEE488=2
; SCSI=3
[INFO]
NAME=CCD_LOCAL

[COMMUNICATION]
TYPE=2
CHAN_NUM=5

; Specifies the controller type
; HC135=509 (Photon counter - Hamamatsu)
; LOCKIN=508 (SR510, SR810)
; SAQ_TRIAX=507 (Direct Drive)
; DS_TRIAX=506 (Direct Drive)
; CCD 3000=505 (IEEE-488 addr 5 only)
; TempBath=504
; CCD 2000=503 (see also [ISA CCD Driver] entry in SYSTEM.INI)
; SPECTRAQ=502
; DATASCAN=501 (DS1010 or CCD3000 External Input)
[CONTROLLER]
TYPE=505

; 2 CTI cards, 2 HV cards and a CCD controller
[PORTS]
NUM_MOT_CHANNEL_PORTS=8
NUM_ACQ_CHANNEL_PORTS=5
NUM_HV_CHANNELS=2
NUM_CONTROL_CHANNELS=0
NUM_LIGHT_CHANNELS=0
; Number of ports is max(8,5,3) --> 8
; mc_ports==> 1..8
; acq_ports==> 1..5
; hv_ports==> 1..2
;
; In api, would create 8 port objects with command channels equal to 0..7
; mc_command_channels 0..7
; acq_command_channels 0..4
; hv_command_channels 0..1
CCDEXP.INI

This file defines the CCD chip size, pixel spacing and linearization parameters for the Spex-3D. This file is instrument specific since linearization is run on each new instrument.

```
[CCD]
XPixels=512
YPixels=512
XSpacing=240
YSpacing=240
;BorderLeftX=0
;BorderRightX=0
;BorderTopY=100
;BorderBottomY=100

[Instrument]
SPC_CenterX=500
SPC_CenterY=500
SPC_RangeX=400
SPC_RangeY=250
PolynomialXC1=-0.00002
PolynomialXC2=0.9877
PolynomialXC3=243.18
PolynomialYC1=0.00008
PolynomialYC2=1.2875
PolynomialYC3=124.57
```
CCDLOAD.INI

This file contains specific information the CCD chip. It contains information on the chip size, pixel spacing, and signal collection. The file also has limits set for shutter opening (exposure time) and allowable temperature range for the chip.

; file: CCDLOAD.INI
; CCD hardware (chip and controller) parameters
1 ; CCD Number
768 ; Base address of RISC board (300 hex = 768 dec)

; Number of pixels
512 ; total_active_x_pixels (in the horizontal direction)
512 ; total_active_y_pixels (in the vertical direction)
15 ; number_serial_pxls_before_active_area
15 ; number_serial_pxls_after_active_area
0 ; number_parallel_rows_before_active
0 ; number_parallel_rows_after_active

3 ; readout register location and direction
; add one from each of the following:
; register location = 0 left
; 1 top
; 2 right
; 3 bottom
;
; direction is relative to our standard configuration
; with the readout register in the left position
; direction = 0 top to bottom
; 4 bottom to top

; 
; /

; ^

; |       |       |       |       |       |       |
; | |ustain---**-> | | | | | |-**
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; * | | | | | | |
; * | | | | | | |
; * | | | | | | |
; 0 | 1 | 2 * | 3
; * | | | | | |
; * | | | | | |
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; * | | | | | |
; ___ | ___ | ___ | ___ * <-****
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; ___ <-**** |
; * |
; * |
; * |
; * |
; * |
; * |
; 4 | 5 | 6 * | 7 |
Limit checking for driver calls

0 ; Minimum temperature in degrees K
300 ; Maximum temperature in degrees K

1 ; Minimum shutter time in milliseconds
400000000 ; Maximum shutter time in milliseconds

0 ; Minimum Gain
4 ; Maximum Gain

; Returned in driver call. Currently not used by the driver.
; pixel spacing/size in tenths of um
240 ; horizontally
240 ; vertically