



Biomedical, Inc.

# Model 410 Circular Dichroism Spectrophotometer

## Instrument Manual

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CDS version 3.20  
AMC version 1.12  
revised 9/2009



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One year from date of shipment.

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# Chapter 1: General Description and Specifications

## 1.1 Introduction

The AVIV Model 410 is a spectrometer for the precise automatic recording of Circular Dichroism (CD) signals. Care has gone into the design and construction of the Model 410 to make it the premier CD instrument in the world.

The Model 410 can functionally be divided up into several subsystems. Each subsystem is required for proper instrument operation. A detailed description of each subsystem is included in this section to acquaint the operator with the instrument.

This section is intended to give an explanation of how the instrument operates. A far more concise explanation of how users should operate the instrument is given in the "Use of the Aviv Model 410 Circular Dichroism Spec-

trometer". Inexperienced users should rely training by an experienced user or by Aviv Biomedical.

The Model 410 can be divided into subsections that together make a whole instrument. The subsections: optics, electronics, mechanics, and software. The software description is discussed in the "Software Details" chapter, as well in sections on the accessories and experiment design.

## 1.2 Theory

A CD spectrometer is the same as a conventional spectrometer, except that additional optics have been added to create circularly polarized light (see Figure 1.1.; Block optical layout). At this stage the light is "normal", in

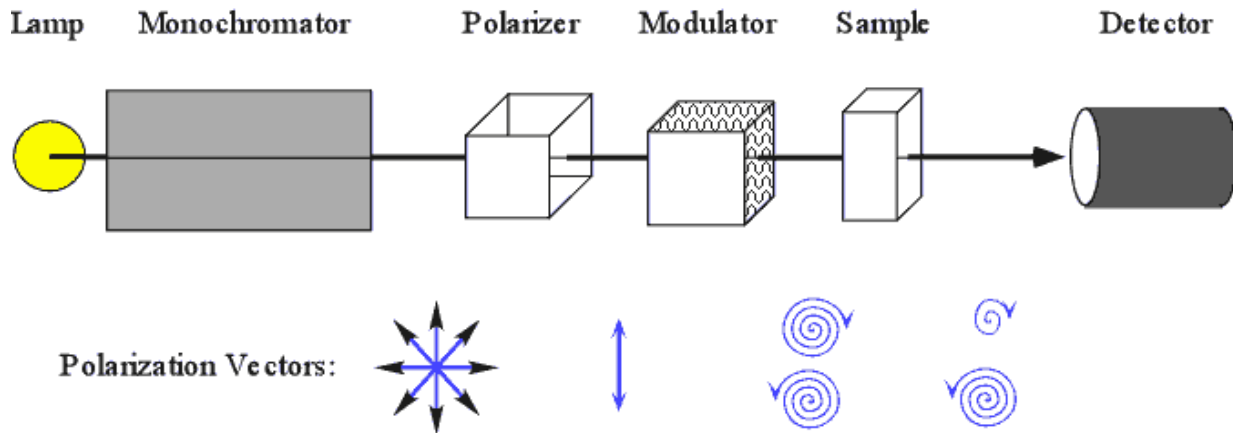


Figure 1.1: Block optical layout

that there is no particular polarization. Light has opposing magnetic and electronic vectors, although only the electronic vectors will be shown. The light passes through a polarizer to produce separate beams of vertically and horizontally polarized light in diverging paths (see Figure 1.2.; Polarization of light). One polarization is discarded. The resulting linearly polarized passes through a photoelectric modulator

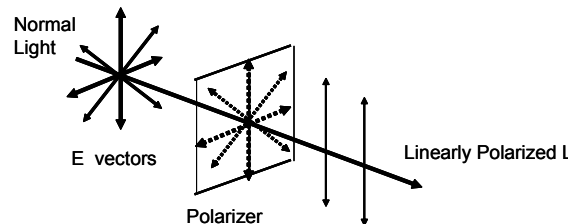
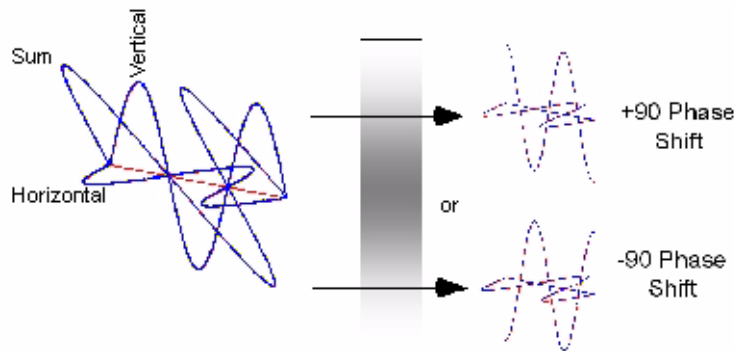


Figure 1.2: Polarization of light

mounted at a 45 degree angle relative to the light's polarization. The modulator consists of two fused silica blocks: a clear portion that the light passes through, and a metal plated block that is attached to the clear block. The coated block acts as a large piezoelectric oscillator, which produces pressure waves at a frequency

of 50,000 Hertz that travel the length of the two blocks. The pressure waves result in birefringence that shifts the refractive index in the vertical and horizontal directions relative to the modulator (see "Figure 1.3: Effect of photoelastic modulator on polarized light"). When



**Figure 1.3:** Effect of photoelastic modulator on polarized light

the linearly polarized light enters the modulator, from the perspective of the modulator there are equal portions of vertical and horizontal light in phase with each other. The two refractive indices results in a phase shift in the vertical and horizontal polarizations when the light emerges from the modulator. The pressure waves are constantly passing back and forth through the glass, so the phase shifts periodically between positive and negative 90 degrees. When the phase shift is 0 degrees the light emerges linearly polarized. When the phase shift is +90 degrees the sum of the two polarizations produces a spiral that when viewed as it approaches appears to turn to the left (see "Figure 1.4: Left and right circularly polarized lights"). In other words, at time point 0 the vertical vector is full positive and the horizontal is zero. At time point 1 the vertical is now zero and the horizontal is full positive. At time point 2 the vertical is full negative and the horizontal returned to zero. At time 3 the horizontal is negative and the vertical zero, and by point 4 the vertical has returned to full positive and the horizontal back to zero. The resulting

sum of the two vectors describes a circle that turns to the left. For right polarized light the negative phase shift has the circle turning to the right.

The circularly polarized light from the modulator then passes through the sample that can selectively absorb the left or right lights. The light intensity is then measured by a photomultiplier tube. The resulting electrical signal is sent to specialized electronics then pick out the intensities of the left and right polarized lights, along with the total light intensity. The electronics have as outputs electrical signals proportionate to difference between the left and right intensities, as well as the total light intensity. These are sent to the computer software, which takes a divides the ratio signal by the total signal and scales it to give the final CD signal.

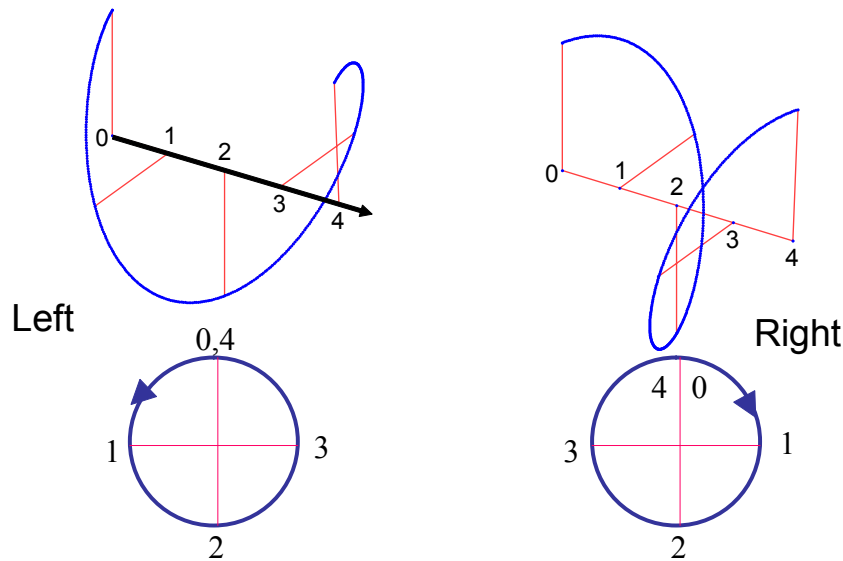


Figure 1.4: Left and right circularly polarized lights

### 1.3 Optics

#### A. Lamp Power Supply

A 150 Watt, air-cooled, Xenon lamp is powered by the Lamp Power Supply. The front panel of the Supply has several lights and displays that show the instrument's status. On the left is an **HOUR** meter, which shows the cumulative hours that the lamp has been on. The **LAMP IGNITE** button will fire the Xenon lamp when conditions are ready. The **LAMP READY** light indicates when the lamp is ready to run. The **INST PWR ON** light shows when power to the instrument is turned on. If the instrument power is on then firing of the lamp is inhibited to prevent damage to the instrument. The instrument power must be off, and the lamp power on, to fire the lamp. **LAMP TEMP ON** shows that the lamp house temperature is within normal limits. If the temperature exceeds a safe value then the lamp will be automatically turned off. The **N2 FLOW OK** light shows that the Nitrogen gas pressure is sufficiently high to operate the instrument. A loss of gas pressure will first sound an alarm for approximately 30 seconds, then turn off the lamp to prevent damage from ozone.

#### B. Monochromator

The white light produced by the Xenon lamp is dispersed by the monochromator. The monochromator contains two (2) fused silica prisms in series. This "w" double monochromator design produces better wavelength resolution and has less stray light than a single monochromator. The prisms provide excellent dispersion of light in the UV. The monochromator has a wavelength range of 165 nm to 1200 nm.

A linkage to a wavelength cam connects the two prisms. The cam converts the non-linear dispersion of the fused silica prisms, into linear motion of the external gear drive mechanism. A computer controlled stepper motor moves the gear drive. The slow speed of this motor is approximately 60 nm per minute. Each motor step corresponds to a 0.05 nm change in wavelength. This step size sets the limit to which wavelength can be specified.

The monochromator drive mechanism is only designed to be accurate when moving from longer to shorter wavelengths. When the program moves the mechanism from a shorter to a longer wavelength, it always overshoots

by 10 nm, and then moves back in order to eliminate backlash in the drive mechanism and restore accuracy.

The linear wavelength scale means that only one point needs to be specified for wavelength calibration. This calibration wavelength corresponds to the position of an

The linear wavelength scale means that only one point needs to be specified for wavelength calibration. This calibration wavelength corresponds to the position of an optical beam switch linked to the motion of the wavelength cam. The program finds the switch position at startup, and associates this position with a calibration wavelength obtained from a disk file. All other wavelengths are determined by counting steps from this point. Wavelength calibration is discussed in detail in the Maintenance chapter.

The monochromator has two sets of sharp edge slits: entrance and exit. The two monochromator slits all move synchronously under control of an external drive system. The width of the slit opening selects the portion of the spectrum sent to the sample. A potentiometer on the drive is sampled by the electronics to sense the slit position. The potentiometer voltage is converted to width and is displayed on the control screen.

A stepping motor is used to move the slits to the desired width. The maximum slit opening of 3.6 mm corresponds to different spectral bandwidths at different wavelengths. In the constant-bandwidth mode, the program converts the desired spectral bandwidth to a slit width using a stored version of the monochromator dispersion function. The slit width shown is in millimeters while the bandwidth is in nanometers.

### **C. Polarizer Compartment**

An achromatic lens is used to focus the monochromatic light from the monochromator exit slit into the sample space. The achromat design focuses the light in the same location, independent of wavelength.

The light is linearly polarized by a  $\text{MgF}_2$  polarizer. The polarizer creates two light beams, of which only the ordinary beam is used in the CD measurement. The extra-ordinary beam is removed by a mask after the modulator.

The linearly polarized light is passed through a photoelastic modulator. The modulator is driven by a 50 kHz oscillator to produce a strain-induced birefringence that is oriented at 45 degrees to the axis of the linearly polarized light. The amplitude of the oscillation is controlled so that the PEM alternately produces +90 and -90 degrees retardation, along one birefringent axis at the oscillation peaks. Thus, the light becomes alternately left and right circularly polarized.

### **D. Sample Compartment**

The sample compartment has been designed to be large to accommodate a variety of accessories. The sample compartment has access through the top, front and possibly on the bottom. The front access is through a removable door/shelf combination. The bottom access hole allows use of the Aviv model 305 stopped flow system. Optional temperature control systems fit inside the sample space, and allow data collection over a wide temperature range. As the light beam is focused on the sample cell, micro cells can be used without special accessories.

### **E. Detector Compartment**

Light is detected by a high speed, high sensitivity end-on photomultiplier tube. A preamplifier, in the PMT housing converts the tube output current to a voltage, which is sent to the electronics for processing.

The PMT is normally operated in a constant current mode. The applied dynode voltage is varied by the software, as necessary, to keep the PMT current constant as the light energy changes.

Dynode voltage for the PMT is produced by a DC to DC converter, and is then applied to a voltage divider chain at the tube socket. The high voltage generator is mounted near the PMT housing. The generator produces about 280 volts for each volt of input from the D/A converter. The high voltage output is sampled by the electronics and is displayed on the control screen.

## 1.4 Specifications

Specifications are subject to change. Consult the your Sale's Quotation for your instrument's specifications.

### A. Optical Performance

Wavelength Range:

170 nm to 875 nm with standard PMT.

Stray Light:

<0.001% at 200 nm

<0.1% over operating limits.

Wavelength Accuracy:

170 nm to 300 nm: +/- 0.2 nm

300 nm to 500 nm: +/- 0.3 nm

600 nm to 875 nm: +/- 1.5 nm

Wavelength Repeatability:

500 nm to 600 nm: +/- 0.5 nm

170 nm to 300 nm: +/- 0.05 nm

300 nm to 500 nm: +/- 0.10 nm

600 nm to 875 nm: +/- 0.20 nm

Wavelength Resolution:

200 nm - 0.03 nm/mm

350 nm - 0.05 nm/mm

700 nm - 3.5 nm/mm

Wavelength Drive:

Software selectable wavelength step. Slew speed is approximately 900 nm/min.

Slit Program:

Constant bandwidth from 0.01 to 12 nm. Automatic slit closure during melting experiments to protect sample and optics from UV light.

Baseline Drift:

Less than +/- 0.1 millidegrees per hour drift after 30 minute warm-up.

Baseline:

Baseline subtraction using separate data set.  
PHOTOMETRIC ACCURACY:  $\pm 0.01$  AU (0.005 typical).

Full Scale Hardware Range:

$\pm 5$  millidegrees to  $\pm 1$  degree.

Sensitivity:

0.001 millidegrees to 0.2 millidegrees (depending on hardware range).

RMS Noise:

<0.06 millidegrees at 185 nm

<0.05 millidegrees at 200 nm

<0.04 millidegrees at 500 nm

1.0 nm bandwidth, no sample, with (true exponential) 4 second time constant.

Noise Reduction:

By integration and/or repetitive scanning.

Light Source:

150 Watt Suprasil Xenon lamp controlled by high stability, constant current, DC power supply.

Sample Compartment:

Open ended for mounting of available sample accessories.

### B. Experimental Modes

**Wavelength Step Scan:** Records CD versus wavelength a wavelength range with a fixed wavelength step size.

**Wavelength Schedule:** Records CD versus wavelength using a user defined schedule. Bandwidth and averaging time are fixed.

**Kinetics:** Records CD versus time at fixed temperatures or vs. a temperature ramp.

**Macro Language:** Uses a macro program to control the instrument's operation. A macro editor allows the user to edit existing programs or create new ones.

**Temperature Scan:** Records CD versus temperature using rising or falling temperature steps. Measurements are made at a fixed wavelength. This experiment mode requires a thermoelectric cell holder accessory.

**Temperature Schedule:** Records CD versus temperature using a user defined schedule. Measurements made at a fixed wavelength. This experiment mode requires a thermoelectric cell holder.

**Titration:** Repeated injections of titrant under computer control. Possible experiments are ligand titration and solute induced denaturation. Handles large volume exchanges. This experiment mode requires the titration accessory.

**pH Titration:** Records CD versus pH. pH is recorded via pH electrode in sample. Feedback loop gives given pH steps. This experiment mode requires the pH and titration accessories.

### C. Data Analysis

Direct transformation, all common math operations.

Fitting straight line, linear regression.

Statistics residuals, standard deviation, mean, median.

Baseline correction, molar ellipticity, and absorbance calculations.

### D. Environmental Requirements

The spectrometer should be housed in a location moderately ventilated, free of dust and excessive mechanical vibrations. The CD spectrometer can operate in the range between 15 and 30°C. As the instrument is a moderate heat source, it is desirable to determine if there is adequate room temperature control. It is possible to calculate the heat output of the instrument

1. **\*\*RECOMMENDED\*\*** Liquid nitrogen type GP45 with built in evaporator, 35 psi minimum pressure required. Please use a high pressure dewar with pressure building capability.

2. Pre-purified, dry nitrogen (tank color code TAN) water pumped.

NOTE: Using this method supplies the instrument for approximately one (1) day or eight (8) hours

### E. Installation and Setup

All installations are performed by Aviv Engineers, except in some countries where Aviv agents will install the instrument.

The initial installation and setup of the instrument is included in the purchase price of the instrument. The optical components in the polarizer compartment are removed, packed and shipped separately. The Xe lamp is packed in the original packing from the manufacturer. The external power supply and any options are packed in their own cartons.

### F. Computer and Peripherals

The computer, monitor, keyboard and printer are each packed in their own carton as provided by the manufacturer. Instructions for packing and setup are included in the computer User's manual. The cartons for the computer components should be retained in the event that the component must be returned to Aviv for service.

### G. Software Installation

The Aviv main program, called CDS, is loaded on the hard drive in a directory C:\AVIV\CDS.

Back up software is provided on a floppy disk.

There is no need to install any software into the computer of a new instrument.



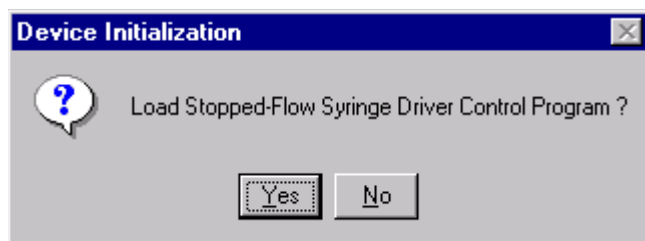
## Chapter 2: Software Details

This chapter provides a detailed explanation of each input to the CDS software. An explanation of how to run the instrument is given in the chapter entitled Quick Start. Version 3.20 of CDS was used for this chapter, which may be different from the version installed on your CD instrument.

### 2.1 Starting the Software

When the instrument control software is started it first checks the individual pieces of hardware to ensure their functioning. Once this is completed the Main Control Panel is displayed. It is here that the current instrument readings are shown along with data from experiments in progress. If the instrument is equipped with the Stopped-Flow accessory then a message will appear that asks if the motor controller program should be loaded. Under most

**Figure 2.1:** Start-Up Message



circumstances the appropriate answer is Yes. The only time No should be used is if it is undesirable to erase the current motor positions from memory.

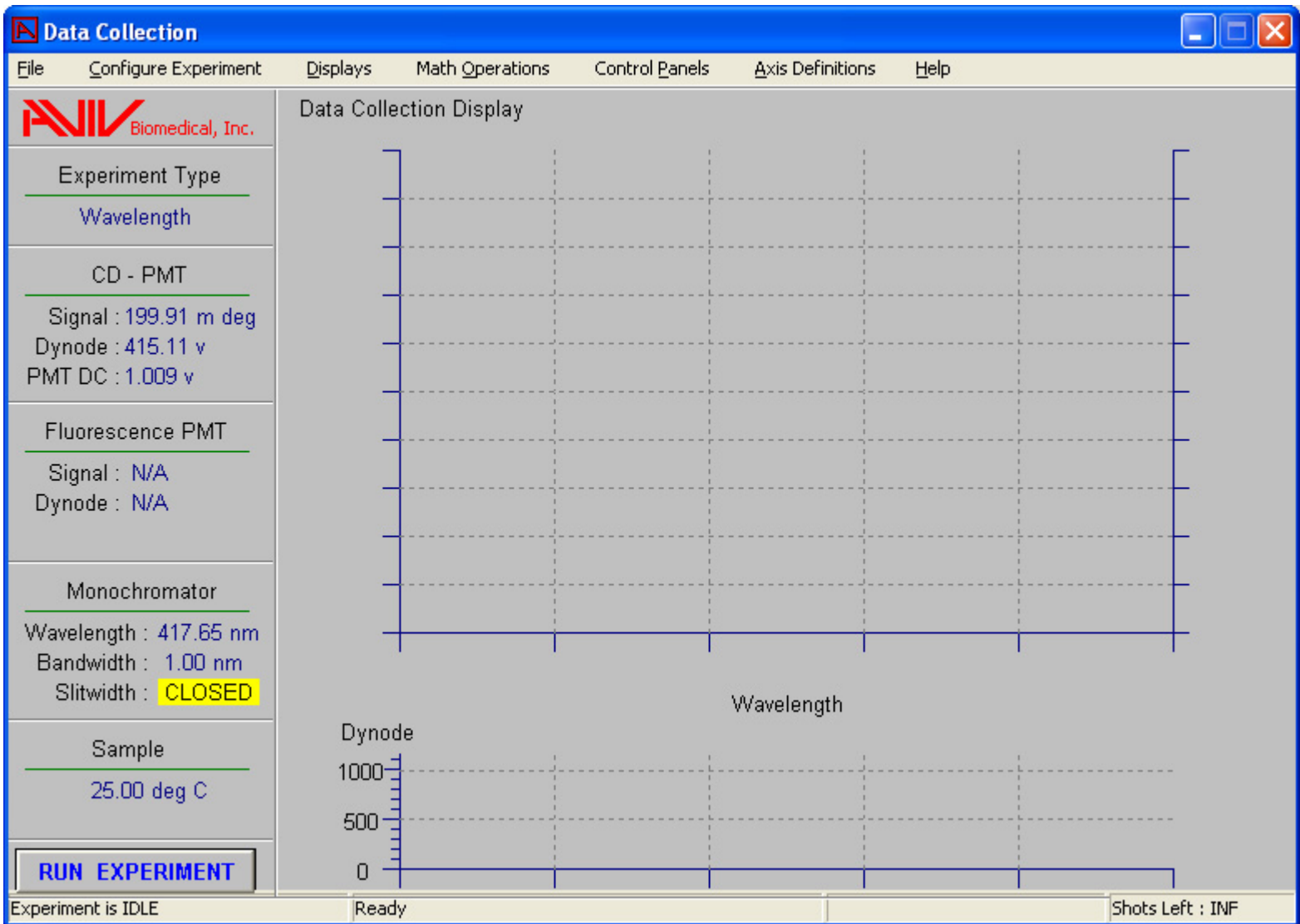
Progress of the start-up procedure is shown in the bottom line of the Main Control Panel. If an initialization test fails then an error message will be displayed. These are explained in “Chapter 5: Service and Troubleshooting”.

### 2.2 Data Collection Panel

The Main Control Panel first displays the **Data Collection Display**, used to graph the collected data, as well as the current values of the different pieces of hardware. **Experiment Type** is the type of experiment that will be executed when the **Run Experiment** button is clicked on. **CD-PMT** is the in-line photomultiplier tube (PMT) used to measure the light transmitted through the sample. **Signal** is the current CD signal, **Dynode** is the high voltage applied to the dynodes of the PMT and **PMT DC** is the current through the PMT. One volt on the PMT-DC display corresponds to 500 nano-amps of actual current. Instruments equipped with a fluorescence accessory will show the current values for the **Fluorescence PMT**. **Signal** is the relative fluorescence signal (full scale is 0 to 5) and **Dynode** is the high voltage applied to the dynodes of the fluorescence PMT. Lines beneath **Monochromator** show the properties of the exciting light. **Wavelength** is the current selected wavelength. **Bandwidth** is the range of light wavelengths that are passing through the monochromator. These values are controlled in the Configure Experiment panel. **Slitwidth** is the mechanical opening between the slits. This value is determined by the user selected values of the Wavelength and Bandwidth. Beneath **Sample** are the current readings for the temperature, pH and other items.

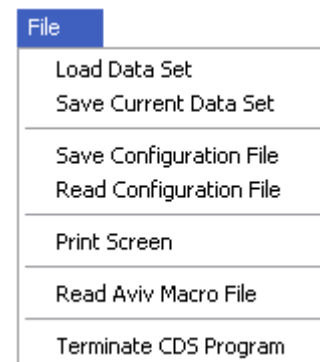
The **File** menu (“Figure 2.3: File Menu”) has options for reading and saving the different types of files. **Load Data Set** opens the Data Browser, where Read Data Set <- Disk can be used to load data into program memory. **Save Current Data Set** gives the user to save data currently displayed in the Data Collection Display. Saving of other data sets is done through the Data Browser (see section “A. Data Browser”). **Save Configuration File**

Figure 2.2: Main Control Panel



allows the current experiment configurations to be saved to a file. This file includes the parameters for all of the experiments. **Read Configuration File** allows the configuration to be set using a configuration file already saved. The reading and writing of data files is done through the Data Browser, found under the Displays menu. Read Aviv Macro File opens the dialog box for reading macro file programs. This is explained in greater detail in “Chapter 6: Macro Language”. **Print Screen** launches a subprogram that will send the image of the current panel printer. The subprogram can be

Figure 2.3: File Menu

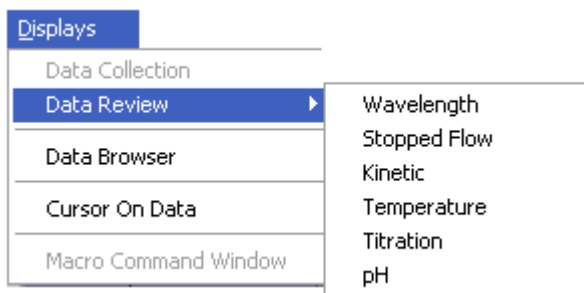


minimized and other panels brought forward for printing. **Read Aviv Macro File** loads a macro file into program memory so that it becomes available in the Experiment Type List. **Terminate CDS Program** exits the program. If data has not been saved then the user will be given a last chance to save data before it is lost.

The **Configure Experiment** menu option opens the Configure Experiment panel. This is where the experiment type and other parameters are selected. This explained later in “Section 2.3 Experiment Configuration”.

The **Display** menu allows the user to select from between two displays of the data. The **Data Collection** display (first seen when the software is started) graphs data as it is being collected, or shortly after a completed experiment. It also includes a separate graph of the dynode voltage at the bottom. The **Data Review** panels allow displaying data from older experiments that are loaded into the program memory. The Data Review submenus select the type of data to be displayed in the Data Review display. The **Data Browser** menu

**Figure 2.4:** Display Menu

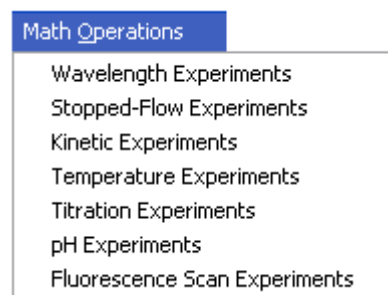


option opens the Data Browser, which allows the user to read and write data files, examine the raw numbers of a data set, and other file management options. These are explained in greater detail later in the Reviewing and Managing Data section. **Cursor On Data** turns on and off a cursor that sits on the data point closest to the mouse arrow. The bottom, left,

and right axis coordinates of the cursor are shown at the bottom of the Data Collection and Data Review panels. If the cursor is turned off then the coordinates are for the mouse position on the graph. The **Macro Command Window** menu option becomes available when a macro experiment has been loaded into CDS. Selecting it opens a panel that displays the commands in the currently loaded macro program.

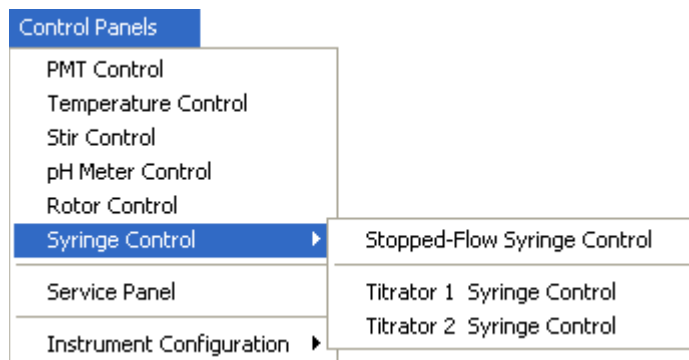
The **Math Operations** menu opens the Data Operations panel for performing math operations. The user must select the type of data that is to be used.

**Figure 2.5:** Math Operations Menu



The **Control Panels** menu (“Figure 2.5: Math Operations Menu”) allows the user to manipulate the individual pieces of hardware. These are explained in detail in “2.7 Control Panels”. The **PMT Control** allows the high voltages of the photomultipliers to be set. **Temperature Control** allows the user to set the temperature set point, equilibration time and other parameters associated with temperature control. **Stir Control** allows the user to turn stirring on and off and to set the speed. **pH Meter Control** reads back the current pH values from the pH accessory. **Syringe Control** reads back the current volumes of the syringes, set the syringe sizes, and other syringe functions. This section is further subdivided between the **Stopped-Flow** syringes, **Titration Syringe** pump 1, and **Titration Syringe** pump 2. **Service Panel** contains the current software version, a control for autoscaling the data and

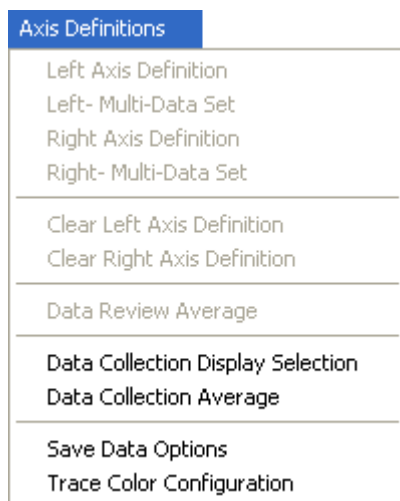
Figure 2.6: Control Panels Menu



instrument utility functions. **Instrument Configuration** contains additional menu options for configuration of the hardware.

The **Axis Definitions** menu allows the user to control display of the data. The first seven options apply only to the Data Review display (greyed out in "Figure 2.7: Axis Definitions Menu"), while the next two are for the Data Collection display only. **Data Collection**

Figure 2.7: Axis Definitions Menu



**Display Selection** allows the user to turn off or on the display of the different types of data being collected. **Data Collection Average** allows the user to select and save data at the end of an experiment for averaging (see description for Data Review Average). **Save**

**Data Options** selects the different types of data to be saved during future experiments. **Trace Color Configuration** allows the user to change the colors of the data being displayed.

The **Help** option in the Help menu brings up on-line help file. The **About** option displays information about the Aviv company and the software version history.

Figure 2.8: Help Menu



## 2.3 Experiment Configuration

This is the location in which the current experiment parameters are displayed and changed. The **Configure Experiment** panel ("Figure 2.9: Configure Experiment Panel") allows the user to select the experiment type, name, and current instrument parameters. In the panel itself, the **Description** is where the user can enter any descriptive information about the experiment. This can include the sample's contents, preparation, lot number, or what ever else is useful in identifying the experiment. Under the general heading of **Experiment** are the parameters that will be used for the next experiment. **Name** is used to name the experiment in the program's memory (as seen in the Data Browser) or on disk. This is followed by the **experiment number**. The number is incre-

Figure 2.9: Configure Experiment Panel

mented every time an experiment is started and then appended to the experiment Name. This approach ensures that every experiment has a unique name and yet doesn't have to be manually changed. Beneath Name is the **Data Set Path**, the location on disk that the experiment will be saved to. The path can be changed in the Data Browser. **Type** is where the user changes the kind of experiment that will be ran next. Beneath Type are the current experiment parameters. These can be changed by going to the Experiment Configuration menu, discussed later.

Under the general heading of **Instrument Status** are the current instrument parameters. First is **PMT Control** with the button **Set Voltage**. Clicking on this brings up the PMT Control panel. If the instrument is in the Stopped-Flow experiment mode then **Time Constant** appears. This has a pop-up list of

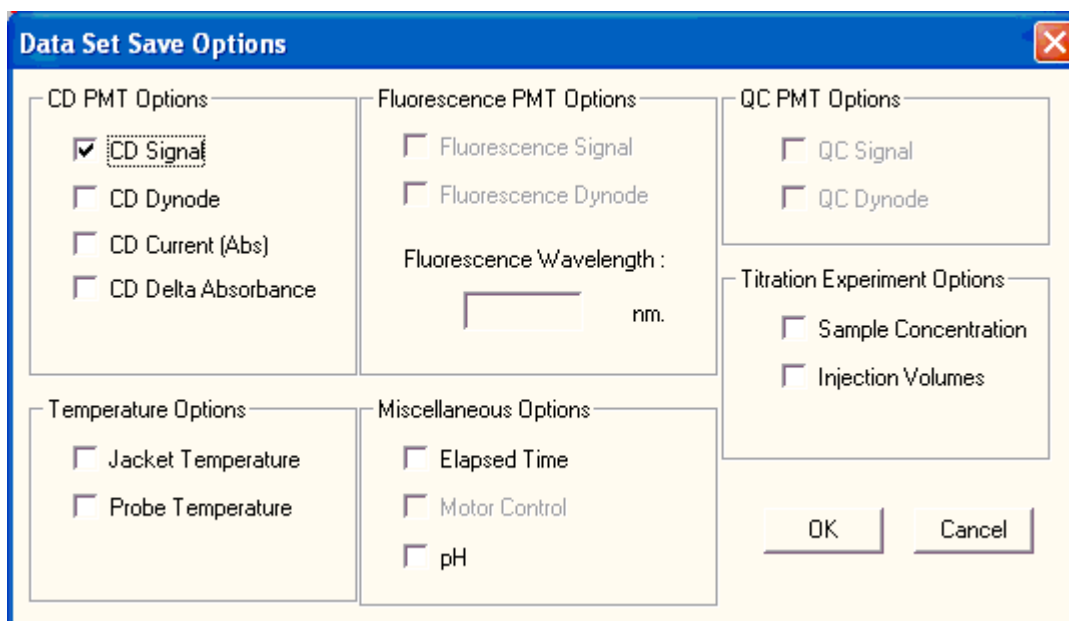
the available electronic time constants. If the experiment type is Stopped-Flow then the default will switch to 100 microseconds when the Experiment Configuration panel is closed. For other experiments types the default is 100 milliseconds. Under **Monochromator/Slits** are **Wavelength** and **Bandwidth**. If these values are changed then the instrument will immediately go to these values when the Return key is hit. When the instrument first starts it goes to the home wavelength, in the range of 530 nm. The Bandwidth determines the peak-half-width for the spectral dispersion of light. A larger Bandwidth allows more light to strike the sample at the expense of spectral resolution (good for highly absorbing samples). A smaller bandwidth passes less light with a narrower range of wavelengths (good for resolving sharp peaks). The bandwidth is controlled by adjusting the slits of the monochromator, as seen in the main control panel.

If the **Auto Slit Closure** option is off then the slits are always left open. If this option is on then the slits will be closed when ever the instrument is idle between experiments, and during equilibration periods longer than 15 seconds. By closing the slits the sample will photobleach less and the life span of the instrument's optics will be extended. Under the heading of **Temperature Control** is the **Setpoint** for the temperature and the current **Deadband**. These are the same parameters as seen in the Temperature Control panel.

The menus in the Experiment Configuration panel are **Exit/Save Configuration**, **Save Data Option** and **Experiment Configuration**. The first closes the Experiment Configuration panel, making the current selections the values for the next experiment. If the panel is closed by using the X in the top right corner of the panel then any changes to the experiment configuration do not take place.

Clicking on the Save Data Options menu item brings up the **Data Set Save Options** panel.

**Figure 2.10:** Save Data Options Menu



This is where the user selects which data is to be saved at the end of the experiment. Under the general heading of **CD PMT Options** are the optical signals. **CD Signal** is the measured Circular Dichroism signal. In all experiment modes except Kinetics and Stopped-Flow this signal is accompanied with the measured standard deviation for the CD signal (CD Error). **CD Dynode** is the high voltage applied to the photomultiplier tube used for CD measurements. The optimal range for this signal is 180 to over 500 volts. Beyond these limits the CD signal can be adversely affected. **CD Current (Abs)** is a signal proportionate to the current

passing through the photomultiplier tube. A signal of 1 equals 50 nanoamps. In most experiment modes (except Stopped-Flow) this value is constant and close to 1. **CD Delta Absorbance** is the raw signal proportionate to the CD signal. This signal is normalized by the CD Current signal and multiplied by 100 to give the CD Signal. Other optical signals may not be available if the software did not detect the necessary hardware at startup.

Under the heading of **Fluorescence PMT Options** the **Fluorescence Signal** is the current on the right-angle fluorescence photo-

multiplier tube. The current is proportionate to the amount of light illuminating the sensor. **Fluorescence Dynode** is the high voltage applied to the right-angle photomultiplier tube. Fluorescence Wavelength allows a different wavelength to be entered for fluorescence measurements (as opposed to CD or absorbance measurements). During Titration, pH Titration and Temperature experiments the wavelength will change, depending on the type of measurement being made.

Under the heading of **QC PMT Options** the **QC Signal** is the current on the quantum counter photomultiplier tube. This measures the total amount of light striking the sample, and can be used for correcting fluorescence and absorbance data. **QC Dynode** is the high voltage applied to this photomultiplier tube. Under **Miscellaneous Options** is **Elapsed Time**. This is the time since the start of the current experiment when the data was collected (in seconds). **Motor Control** is for Stopped-Flow experiments. **pH** allows the pH to be recorded in addition to other types of experiments when this accessory is used. For example, during a Titration experiment, the pH can be monitored while the ligand concentration changes.

Under the heading of **Temperature Options** are Jacket Temperature and Probe Temperature. The physical location of these sensors depends on the type of thermoelectric accessory being used, but in general the **Jacket Temperature** is the probe used for the thermal feedback loop, and is the temperature closest to the actual sample temperature. The **Probe Temperature** is usually associated with the external temperature probe accessory. This can be inserted into the sample to allow measurements of the sample. For details pertaining to your particular thermoelectric accessory see Appendix A.

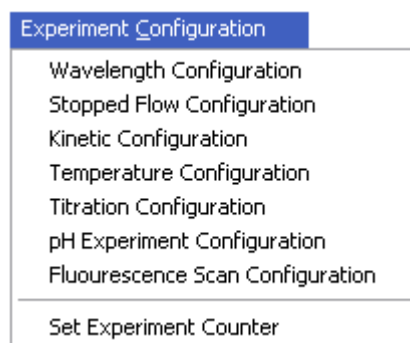
**Miscellaneous Options** has a collection of all the other data types. **Elapsed Time** is the time that has passed since the start of the experi-

ment. **Motor Control** is a record of the transitions between different time domains in Stopped-Flow experiments. **pH** is a record of the pH (assuming the pH accessory is available).

The **Titration Experiment Options** heading has **Sample Concentration**, which is the calculated sample concentration during a titration experiment. The data can be divided by the Sample Concentration to correct for dilution effects and normalize the signal. **Injection Volumes** is injection volume between Titration and pH Titration data points.

The **Experiment Configuration** menu gives access to the configuration panels for each of the experiment types. This is where the details of each experiment type can be modified. **Reset Experiment Counter** resets the number appended to the experiment Name.

**Figure 2.11:** Experiment Configuration Menu



## 2.4 Experiment Configuration Panels

The Wavelength Configuration panel allows the user to enter the wavelength at which the scan will start at (**Wavelength Start**), the wavelength the experiment will end with (**Wavelength End**), the size of the wavelength step (**Sample Every**), and the number of wavelength scans that will be made (**Number of Scans**). At each wavelength the signal will be averaged for the **Averaging Time**. If the averaging time is greater than 0.1 seconds then a standard deviation (error) will also be calculated for each data point. **Wait Time**

Figure 2.12: Wavelength Configuration Panel

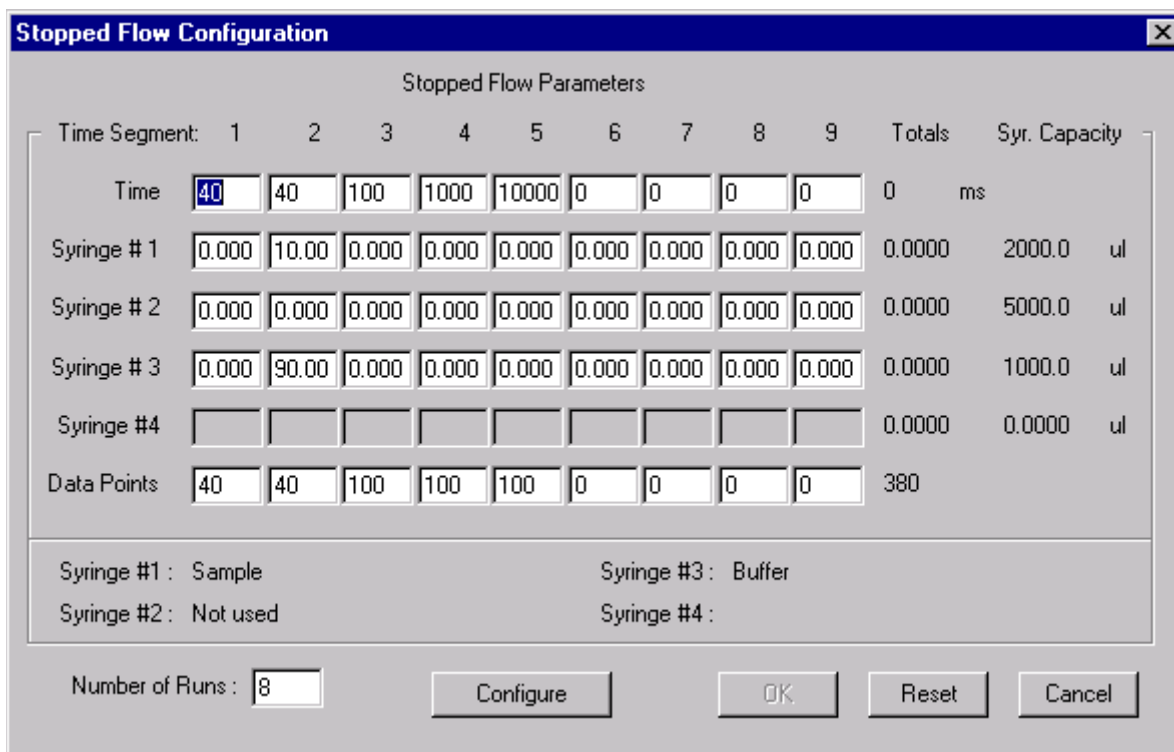
**Between Scans** applies when more than one scan is made. This is an additional pause between wavelength scans, and is useful when long-term kinetic changes are being monitored.

The **Stopped-Flow Configuration** panel allows the user to define the shot size, timing, the number of data points, and the number of shots for stopped-flow experiments. The equipment for this is available as an accessory for the CD instrument. The experiment is divided into nine **Time Segments**. The duration (or **Time**) of each Time Segment is entered in the top row as milliseconds. During each Time Segment the volume in microliters to be delivered by each syringe is entered (**Syringe #1, #2, #3**). The ratio of the volume to time determines the flow rate for each individual syringe, and the sum of the flow rates is the total flow rate passing through the flow cell. Across the bottom is the number of **Data Points** to be collected during each time

segment. Likewise the ratio of the number of data points divided by the time gives the data collection rate. In the example shown the first 20 milliseconds are spent collecting data at a rate of 0.1 milliseconds per data point with no flow (to measure the pre-trigger signal). In the second Time Segment 20 microliters is delivered syringe #1 (the 2 ml syringe) and 80 microliters from syringe #2 (the 5 ml syringe). Therefore the flow rates are 0.5 ml/second plus 4.0 ml/second for a total of 2.5 ml/second through the flow cell. The dilution ratio of the Stock Protein in syringe #1 is 1:4 with buffer, or 1:5 total dilution. The data collection rate continues at 10 data points every millisecond to measure the push profile. In the third Time Segment the high data collection rate continues for 100 milliseconds to measure the rapid kinetics. In the fourth and fifth Time Segments the number of data points is the same but the times are longer, so the data collection rates are 1 and 10 milliseconds per



Figure 2.13: Stopped Flow Configuration Panel

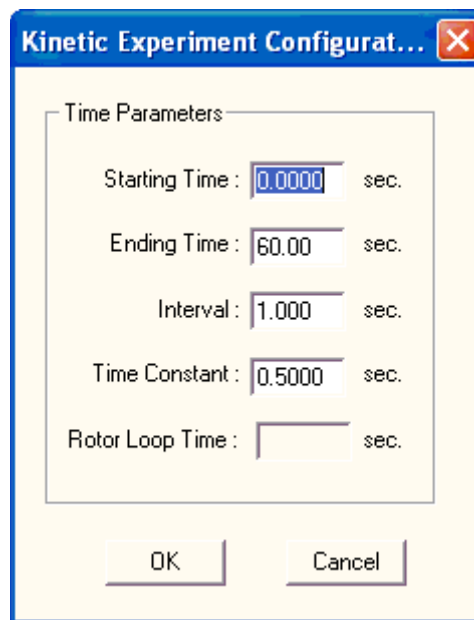


data point. The advantage of collecting data at slower rates is that more signal averaging can occur, and the signal-to-noise ratio will improve. By changing the data collection rates during a single shot the rapid kinetics at the start can be measured (albeit with more noise), then the slow kinetics can be measured more accurately, without creating data files with millions of data points.

The **Number of Runs** sets the number of shots to be made. If one to twenty shots are made then each individual shot is saved with all of the selected data sets. Each run will appear in the Data Browser as a separate data folder within the one experiment folder. If more than twenty runs are made then a running average is kept for all of the data sets plus the data sets from the last run.

The **Kinetic Experiment Configuration** allows the user to measure slow kinetics. The rate of kinetics that can be measured is determined by how fast the instrument can begin

Figure 2.14: Kinetic Experiment Configuration Panel



The limiting factor is usually how fast the sample can be placed in the cell holder and the sample compartment shut. A spring loaded syringe is available as an accessory so that solutions can be manually injected with the sample compartment closed. However, if the kinetics are faster than several seconds then the Stopped-Flow accessory must be used. The **Starting Time** is the time point at which data collection will begin after the experiment has been started. Normally data collection is started with the start of the experiment. **Ending Time** sets the duration of the experiment. **Interval** is the frequency at which data is collected. **Time Constant** sets the response time of the signal. This is done with a software simulation of a RC circuit, and time constant of the signal. For example, if a response time of 1 second is used, then a step in the optical

signal will result in a recorded signal with an exponential decay that at one second has changed 63% of the total. This can be used to optimize the response and noise for the experiment. The response time should be ten times faster than the fastest kinetics that will be measured. This way the response time of the instrument will not interfere with the measurement of the signal, yet there will be sufficient reduction of the noise. The **Rotor Loop Time** sets how frequently the five-position cell holder will be cycled to measure all of the selected cells.

Temperature experiments allow the thermal stability of a sample to be measured. The experiment is performed by the thermoelectric cell holder driving the temperature to a set point. When the is at the set point, plus or

**Figure 2.15:** Temperature Experiment Configuration Panel

**Temperature Experiment Configuration**

**Temperature Parameters**

Starting Temperature : 25.00 deg C

Ending Temperature : 30.00 deg C

Temperature Step : 1.00 deg C

Dead Band : 0.1000 deg C

Equilibration Time : 0.5000 min.

**Options**

Averaging Time : 1.00 sec.

Return to 25 deg C at end of experiment

Reverse Temperature Scan

Reverse Hold Time : 0.0000 min.

**Step Method**

Temperature Step  Temperature Schedule

**Temperature Schedule Parameters**

Add Temperature : [ ] deg C

Edit Temperature

Remove Temperature

Remove ALL

OK Cancel

minus the Dead Band, the Equilibration Time starts counting down. If the temperature exceeds the Dead Band then the clock is stopped and restarted once the temperature re-enters the limits set by the Dead Band. When the Equilibration Time reaches zero then signal are collected for the Averaging Time. When this is complete the temperature set point is incremented to the next temperature and the cycle is repeated. Dead Band and Equilibration Time are set in the Control Panels: Temperature Control panel of the Main Control panel.

The **Starting Temperature** is the first temperature to be measured. Normally it is a low temperature (for a heating scan), but it can be set higher than the Ending Temperature for a cooling scan. **Ending Temperature** is the last temperature to be measured. **Temperature Step** is the size of the temperature increment between optical measurements. **Averaging**

**Time** is the time the optical signal will be averaged to create the data point. If the check box labeled **Return to 25 deg. C at end of experiment** is turned on then the temperature will automatically be set to 25 C when data collection is completed. Turning on the **Reverse Temperature Scan** check box will result in two temperature scans; the first with the Starting and Ending Temperatures defining the limits of the first scan, then the second scan with the Starting and Ending Temperatures exchanged. This type of experiment is good for testing the reversibility of the sample's thermal transition. **Reverse Hold Time** is the time held at the Ending Temperature before the reverse temperature scan is started.

Titration Experiments allow the user to control the concentration of a tattering in the cell. This is useful for measuring the effects of ligand binding, solute denaturation, dilution, or some other concentration dependent reaction. This

Figure 2.16: Titration Experiment Configuration Panel

**Titration Experiment Configuration**

**Titrant Concentration Parameters**

Initial Titrant in Cell : 0 M

Titrant Concentration in Syringe : 0.1 M

**Sample Concentration Parameters**

Initial Sample in Cell : 1e-006 M

Sample Concentration in Syringe : 0 M

**Titrant Concentration Schedule**

(molar Concentration)	Domain 1	Domain 2	Domain 3
Target Concentration :	0.01	0.02	0.03
Concentration Step :	0.001	0.002	0.005
Stir Time (Minutes) :	0.5000	0.5000	0.5000

**Equilibrium Parameters**

Enable Approach to Equilibrium

Target Slope (dy/dt) : 0.1

Slope Measurement Timeout : 60.00 sec.

Slope Measurement Time : 10.00 sec.

**Miscellaneous Parameters**

Temperature : 25.00 deg C

Averaging Time : 5 sec.

**Sample Volume**

Increasing  Constant

Initial Cell Volume : 1.800 ml.

OK Review Cancel

experiment requires the syringe pump option. The software will keep track of two concentrations: the Titrant, whose concentration is generally ramped up; and the Sample, whose concentration may decrease due to dilution. The software asks the user for initial concentrations and volumes, as well as target concentrations, then the appropriate injection volumes are calculated. During the experiment the software keeps track of the volumes in the syringes and will automatically refill or empty the syringes at the appropriate times. The order of events is measure the initial optical signals, make an injection, stir the sample, and make the next optical measurement. When this is done the next cycles of injection, stir, and measurement are performed until the experiment is completed.

**Initial Titrant in the Cell** is the concentration of Titrant (i.e., the ligand or denaturant) in the cell. Normally this is zero but it need not be so. **Titrant Concentration in Syringe** is the concentrated solution being injected. It represents the upper limit for the accessible concentration range. **Initial Sample in Cell** is the concentration of Sample (protein, macromolecule, or what ever) in the cuvette. **Sample Concentration in Syringe** is the concentration of the same material that is in the syringe. Frequently this is zero, so the software will calculate the dilution of the sample during the experiment. However, it is possible in some cases to put the same concentration of Sample in the syringe as in the cell. This will eliminate dilution effects of the Sample, making data easier to interpret. However, this can only be done safely if the Sample can withstand extended exposure to the Titrant and still exhibit reversible chemistry. For example, during solute denaturation experiments on proteins the volume of denaturant injected can exceed the volume of the original sample. This can result in an optical signal that changes more from the dilution of the sample than from any chemical transformations that may take

place. If the Sample concentration is the same in the Syringe and Cell then there will be no dilution of the sample during the experiment.

The intermediate concentrations of Titrant are determined by the **Titrant Concentration Schedule**. This is divided into three Domains, each with its own **Target Concentration, Concentration Step**, and **Stir Time**. The Initial Titrant in Cell is the starting concentration for the experiment. The first injection raises the Titrant concentration by the Concentration Step of Domain 1, then the software waits for the Stir Time of Domain 1 before making optical measurements. This repeats until the Target Concentration for Domain 1 is achieved. Then the parameters for Domain 2 are used. The experiment ends when the Target Concentration for Domain 3 is reached. The parameters for the three Domains can be customized for the sample's characteristics. For example, if a solute denaturation experiment is done that has a midpoint at four molar, then the initial baseline is of zero to two molar is moved through quickly. In the transition range of two to six molar the concentration steps are smaller to give better definition of the transition. The stirring time is increased to allow for the slower rate that the sample reaches chemical equilibrium. In the final baseline region of six to eight molar the concentration step is larger and the stirring time shorter to quickly end the experiment.

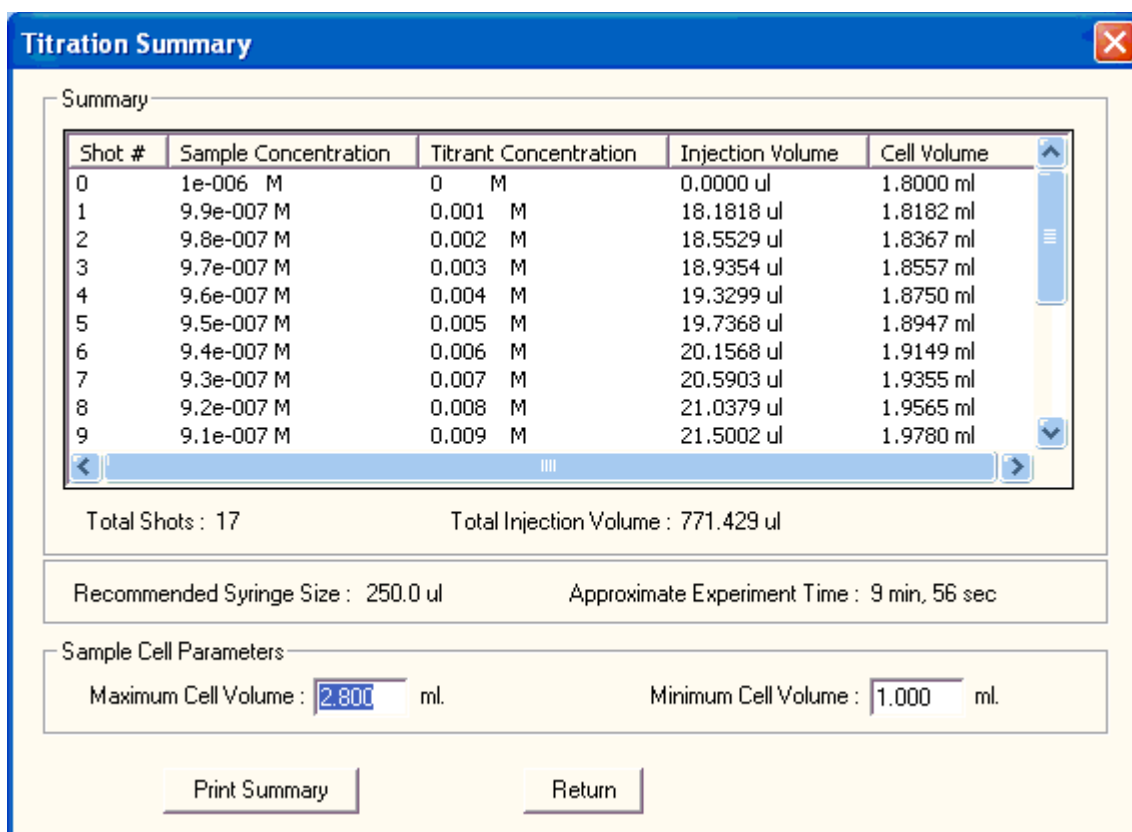
The temperature of the experiment is set in the Main Control panel, Control Panels: Temperature Control panel. The signal is measured for the **Averaging Time** and the standard deviation is calculated for each individual data point. The **Sample Volume** can be allowed to increase (**Increasing** check box) or can be held **Constant**. If Increasing is selected then only injections will be made. If Constant is selected then before each injection the second syringe of the syringe pump will remove an aliquot, then the same volume of Titrant will be injected. This way the same volume is held

during the whole experiment. The advantage of this approach is that the cuvette can never overflow, and the range of accessible Titrant concentrations greatly increases. Without this feature solute denaturation experiments must be broken up into several experiments covering different concentration ranges, then pasted together to obtain the entire concentration profile. When the **OK** or **Review** buttons are selected the software performs several checks of the validity of the experiment. Checks are made for overflowing the cuvette

(Increasing volume mode), underflowing the cuvette (Constant volume mode), and negative injection volumes (concentrations wrong or out of sequence). **OK** accepts the experiment, closes the panel, and make Titration experiments the current experiment. **Return** closes the panel without accepting the changes.

If the Review button is hit the schedule of shots is shown with the calculated volumes and concentrations. Also shown are the Total

**Figure 2.17:** Concentration Summary Panel



Shots that will be made for the experiment, the Total Injection Volume that will be used, the Recommended Syringe Size, and the Approximate Experiment Time. The pump requires another 0.5 ml to fill the lines, so plan on making an excess of Titrant for the experiment. The Recommended Syringe Size is based on the smallest injection that will be made. The

pump uses stepper motors to drive the plungers, which have 2000 steps for the full plunger distance. Therefore the limiting factor on the accuracy of delivery of an injection is how close is the requested volume is to an integer number of steps. Fractional steps are impossible, so anything less than 1/2 of a step is rounded up or down. To minimize this error

the software wants to take a minimum of 100 steps per injection, so it recommends a syringe size that meets this requirement. If the larger injections are larger than the size of the syringe then the injection is broken up into several passes, so the syringe size does not limit the size of the injection. As describe before the software automatically performs overflow and underflow checks. To do this it

must know the **Maximum Cell Volume** and the **Minimum Cell Volume**. **Print Summary** sends the volume and concentration information to the printer.

The pH Experiment Configuration panel allows the user to set up automated pH titration experiments. It requires both the syringe pump

**Figure 2.18:** pH Experiment Configuration Panel

**pH Experiment Configuration**

**pH Parameters**

Ending pH : 2

pH Step Size : 0.1000

Averaging Time : 1.000 sec.

Stir Time : 0.5000 min.

pH Dead Band : 0.0100

**Sample Concentration Parameters**

Initial Concentration in Syringe : 1e-6 M

Initial Concentration in Cell : 0 M

**Volume Parameters**

Constant Volume  Increasing Volume

Initial Cell Volume : 1.800 ml.

Max. Added Volume : 2.800 ml.

**Temperature Parameters**

Sample : 25.00 deg C

Equil. Time : 0.5000 min.

Dead band : 0.1000 deg C

OK Cancel

and pH meter accessories. This type of experiment differs from regular titration experiments in that the injection size is adjusted on the fly to give evenly spaced pH steps. The appropriate injection volume is determined by the buffering strength and volume of the sample, as well as the concentration of acid or base being injected. The pH electrode sits in the sample in the instrument, along with tubing from the pump. A feedback loop is created between the measured pH, the computer, the syringe pump, and the sample. Instead of asking the user for the injection volumes the software has target pH values, and constantly adjusts the injection

volumes of acid or base to achieve the target pH values. This makes experiment design much easier because the user does not have to be concerned about changes in the sample's buffering capacity, yet the experiment gives data at fairly constant pH increments.

The starting pH of the experiment is determined by the pH of the sample when it is placed in the instrument. The **Ending pH** is the pH at which the experiment will end. The **pH Step Size** sets the size of the pH steps at which optical measurements will be made. **Averaging Time** is the duration the signal will be measured to produce a data point and stan-

dard deviation. **Stir Time** is the time between when the final injection is made and the optical measurement is made. 0.5 minutes ensures a homogenous solution, but longer times can be entered to ensure complete chemical equilibrium of the sample. **pH Dead Band** is the allowable error in the pH measurement. If the software can not achieve the requested pH Dead Band, or of the pH has overshoot the set point, then optimal measurements will be made despite the pH Dead Band. The Temperature Parameters are set in the Main Control panel, Control Panels: Temperature Control panel. The **Constant Volume** and **Increasing Volume** determine if the syringe pump should use two syringes (one to remove sample and the other to add acid or base), or only one syringe to make injections. The **Max. Added Volume** is the maximum volume of acid or base that can be added without overflowing the cell. If this value is reached then the software will stop the experiment.

## 2.5 Reviewing and Managing Data

Data that has already been collected or loaded into the program's memory can be graphed by going to the Main Control panel and selecting the **Displays: Data Review** menu (Figure 2.4: Display Menu). The Data Review submenu will show a list of experiment types (to select the X-axis of the graph). When a type is selected the **Data Review Display** appears.

To select data for display use the **Axis Definitions** menu. The options for selecting data that were disabled in Data Collection are now available (and options specific to Data Collection are disabled). **Left Axis Definition** and **Right Axis Definition** menu options select single data sets for either axis. These open the **Data Browser** with the appropriate experiment type folder. To select a data set click on it to highlight it and then click on the **Select Data Set** button. If more than one data set is to be selected the **Left- Multi-Data Set** and **Right- Multi-Data Set** menu options are preferred. These give lists of all of the data that hasn't

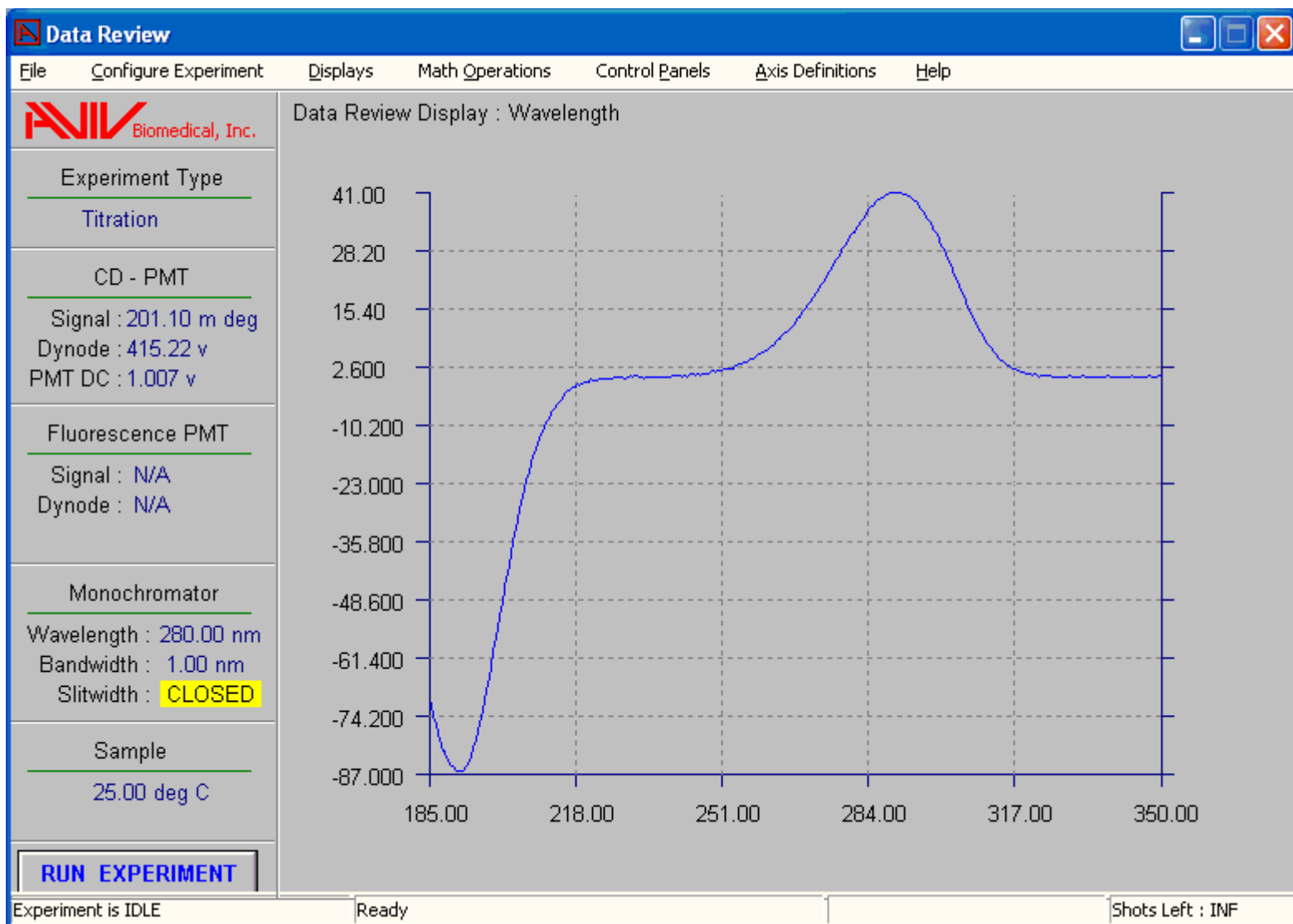
already been displayed. Several data sets can be selected at once for display. **Clear Left Axis Definitions** and **Clear Right Axis Definitions** menu options allow the user to select which data to remove from display. **Data Review Average** is described below. **Save Data Options** opens the Data Set Save Options panel, describe earlier. **Trace Color Configuration** allows the user to define the colors of the displayed data.

The **Data Review Average** panel allows the user to select from the displayed data sets. Data from both the left and right axis can be averaged together (Figure 2.21: Data Review Average Panel). **Experiment** determines the experiment folder in which the new data set will be saved. In Data Collection Average the Experiment input will be the current experiment. In Data Review Average the Experiment input will not include the experiment counter (this must be supplied by the user). **Data Set** is the name of the new data set. When the panel is first opened the user should select the data to be averaged, then push the **Average Selected Traces** button. This calculates the average, displays it in white, but does not save the data. Default inputs are placed into the Experiment and Data Set inputs. **Save Average Trace** saves the average to the Data Browser only (not to disk). **Clear Average Trace** removes the plotted average.

### A. Data Browser

The data stored in the memory of the program is organized in the **Data Browser** (Figure 2.22: Data Browser Panel). This panel is accessed through the Displays menu of the Main Control Panel. The Data Browser only shows data loaded into the program, not data on a hard drive or floppy. If the program is not exited properly then data shown in the Data Browser may be lost if it has not been written to a hard drive or floppy. Several opportunities are given to write data to disk, and safety checks have been placed in the software to check for unsaved data, but no insurance can be made

Figure 2.19: Data Review Display

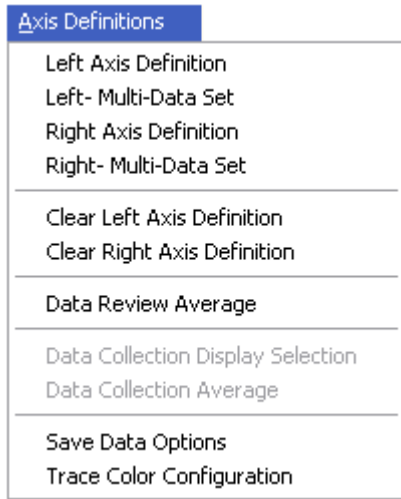


against power loss or inappropriate use of the software. Therefore the safest approach is to save the raw data immediately after it has been collected, and save the data again after calculations have been made. At the end of every experiment an opportunity is given to save the data to disk. Data can be saved to disk at any time with the Data Browser. Finally, when CDS is terminated it warns the user of any unsaved data and gives the opportunity to save.

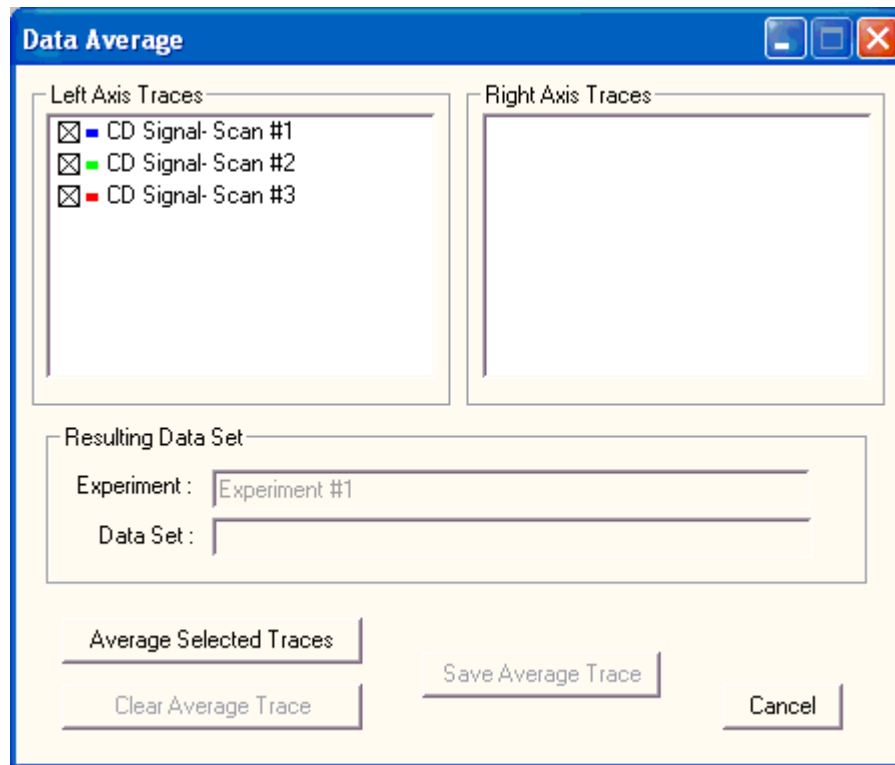
The data is organized in the Data Browser by experiment type. The right half of the panel shows folders all of the experiment types. If an experiment type contains data then a + appears to the left of the type name. Clicking on this (or double clicking on the name) will open the folder. The data is further organized into sub-folders for each experiment. These can be opened in the same manner. In the cases of Stopped-Flow and Wavelength Experiments the experiment folders may contain another layer of sub-folders. These are for



**Figure 2.20:** Axis Definitions Menu for Data Review



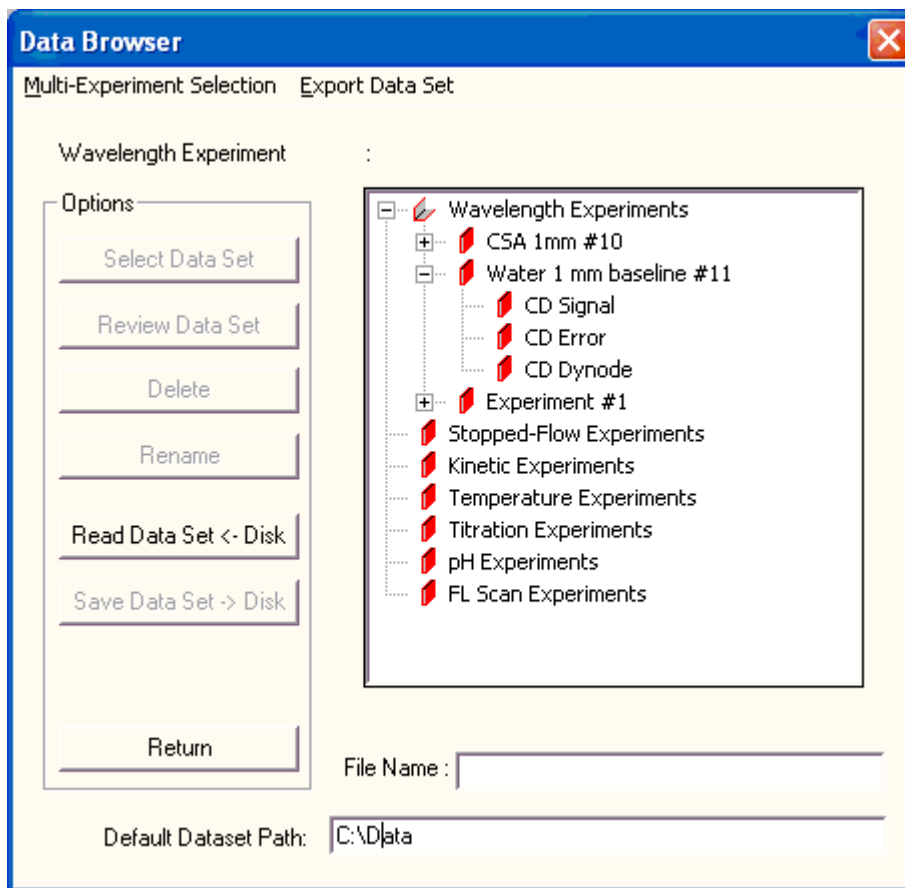
**Figure 2.21:** Data Review Average Panel



each Wavelength scan or Stopped-Flow shot. At the lowest level of sub-folders is the data. Data and sub-folders are selected by clicking on them to highlight the name. Along the left half of the Data Browser panel are buttons for

various operations. The **Select Data Set** button becomes available when data is being selected for display in the Data Collection or Data Review panels. The contents of a data set can be viewed by selecting the data and

Figure 2.22: Data Browser Panel



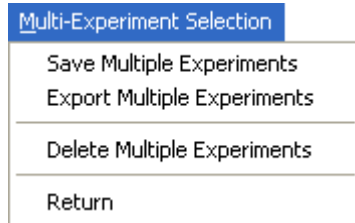
clicking the **Data Review** button. Data and experiment sub-folders can be removed from memory by highlighting on an item and clicking on the **Delete** button. Data and experiment sub-folders can be renamed in the same fashion with the **Rename** button. Experiment files on disk are read into memory with the **Read Data Set <- Disk** button. **Save Data Set -> Disk** button writes the selected experiment folder to disk. The default name for the data file is the name of the experiment with \*.DAT appended. All data is saved in a text format, except for Stopped-Flow data which is binary due to it's large size. If other file formats are needed then use the Export Data Set button (explained below). If a file name different from the experiment is desired the file name should be typed into **File Name** before selecting the Save button. Data is saved to the **Default Data**

**Path**. If a different disk drive or directory is desired then the Default Data Path should be changed. If the new directory does not exist then the user will be given the opportunity to create a new directory on disk. The **Return** button closes the Data Browser panel.

The menu line of the Data Browser panel has several functions. If several experiments are to be manipulated in the same fashion then the **Multi-Experiment Selection** menu can be used to **Save** files, **Export** data to ASCII text files, or **Delete** selected experiments of the same type. **Return** closes the Data Browser panel.

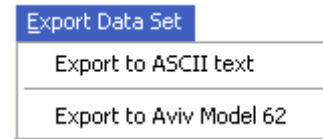
The **Export Data Set** menu has options for saving to other data file formats. **Export to ASCII text** is the best if data must be exported to other programs. File name and data path

**Figure 2.23:** Multi-Experiment Selection Menu



selection is the same as described above. Entire experiments, individual shots or scans, or individual data sets can be saved to text files. If a single data set is selected then the file will contain several headers followed by X- and Y-axis columns of data. Text files use “.TXT” as

**Figure 2.24:** Export Data Menu

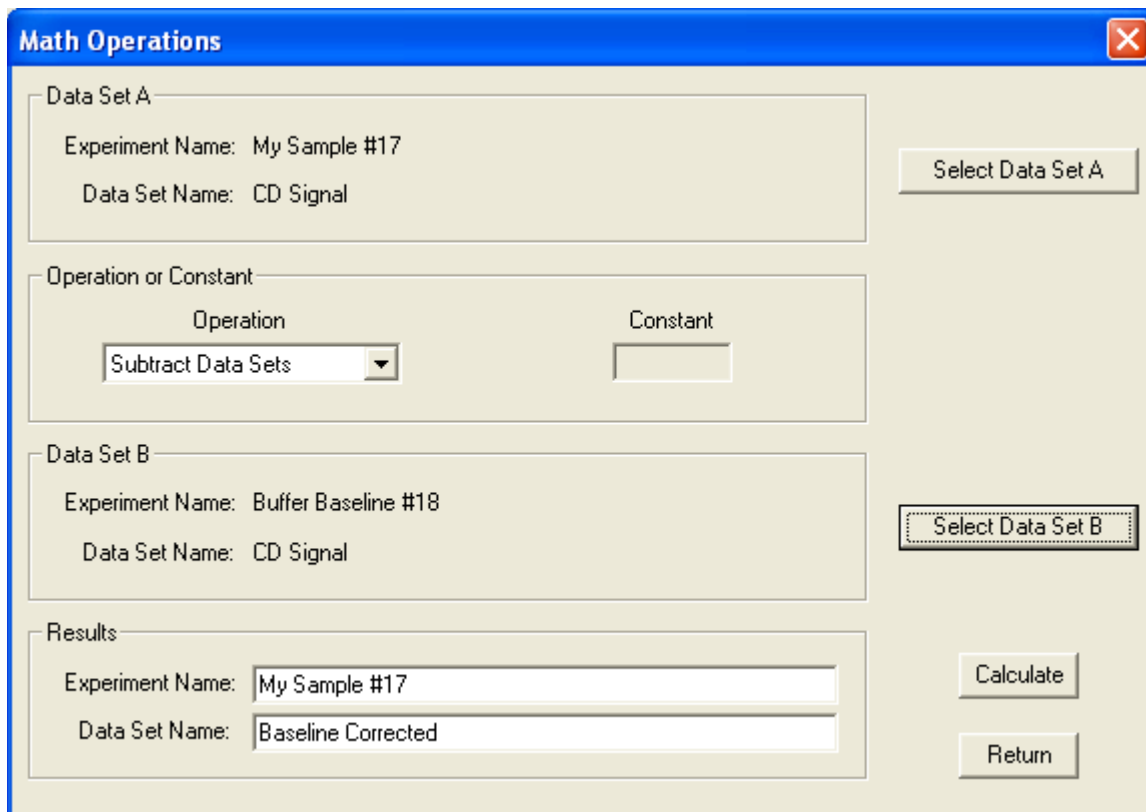


the extension. **Export to Aviv Model 62** saves single experiments to the text format compatible with the Model 62 CD instrument.

## 2.6 Math Operations

Most of the math operations for data are available through the **Math Operations** menu of

**Figure 2.25:** Data Set Operations Menu

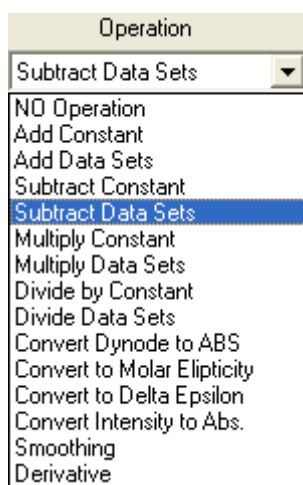


the Main Control panel. The exception is averaging multiple data sets, which is done through the Axis Definitions menu of the Data Collection and Data Review panels. The Math Operations menu gives a list of the experiment types that the user must select from.

To perform math operations the original data set must be selected. Select Data Set A opens the Data Browser, where the user click on a data set to highlight it, click on Select Data Set to make the selection. Next a math operation must be selected. All of the common math

functions are available, both with a constant and between two data sets. If a constant is required it's value should be entered under Constant. If a second data set is needed then it is selected with the Select Data Set B button. The results of the math operation will automatically be placed in the same experiment folder that contains Data Set A. If a different location is desired then the input following Experiment Name should be changed. The user must enter the name for the new data in Data Set Name. When these input have been made the Calculate button becomes available. Clicking on this performs the math operation and creates the new data set.

**Figure 2.26:** Math Operations Menu

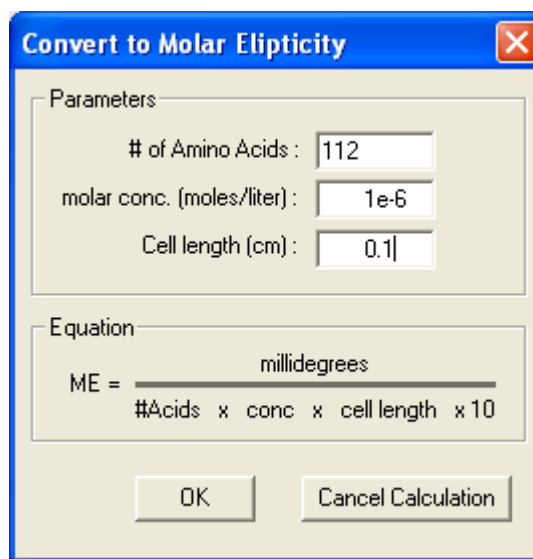


There are several specialized math operations that need more explanation. **Convert Dynode to ABS** estimates the sample's absorption based on the recorded dynode voltage. This works for all experiment types except for Stopped-Flow (see Convert Intensity to Abs. described below). The CD instrument can be used as a single beam spectrometer to record the light intensity. This requires two measurements; one of the sample and one of a reference. The reference determines what absorbance will be calculated. If air is the reference then the absorbance value will include contributions from the sample, buffer, solvent

and cuvette. If the absorbance of only the sample is desired then a cuvette filled with buffer should be used as the reference. The sample should be selected as Data Set A and the reference as Data Set B.

The secondary structure analysis of peptide and protein CD spectra requires that the raw data recorded in millidegrees be normalized to molar ellipticity. The **Convert to Molar Ellip-**

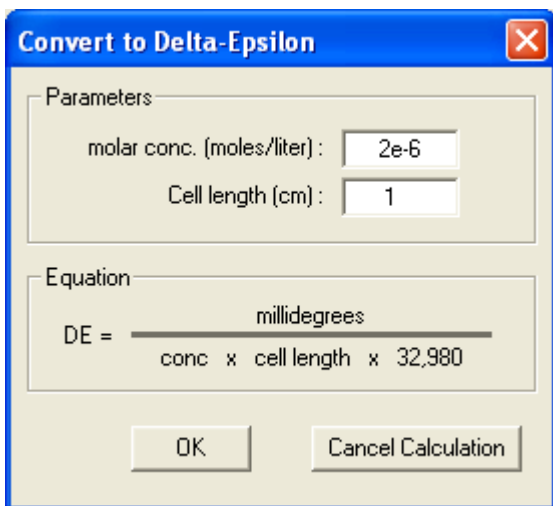
**Figure 2.27:** Convert to Molar Ellipticity Panel



**ticity** math operation performs this function. The normalization is based on the number of peptide bonds in the sample molecule. The normalization allows comparison of spectra taken of small peptides to large protein assemblies. To perform the calculation it is necessary to know the **number of amino acids** in the peptide or protein, the **molar concentration** of the peptide or protein, and the path length of the cuvette (**cell length**).

**Convert to Delta Epsilon** calculates  $\Delta\epsilon$  (Delta Epsilon) based on the millidegrees signal.  $\Delta\epsilon$  is the difference in the extinction coefficients between the left and right circularly polarized light. It normalizes data based on the sample concentration and path length.  $\Delta\epsilon$  has the same units of measurement as the more

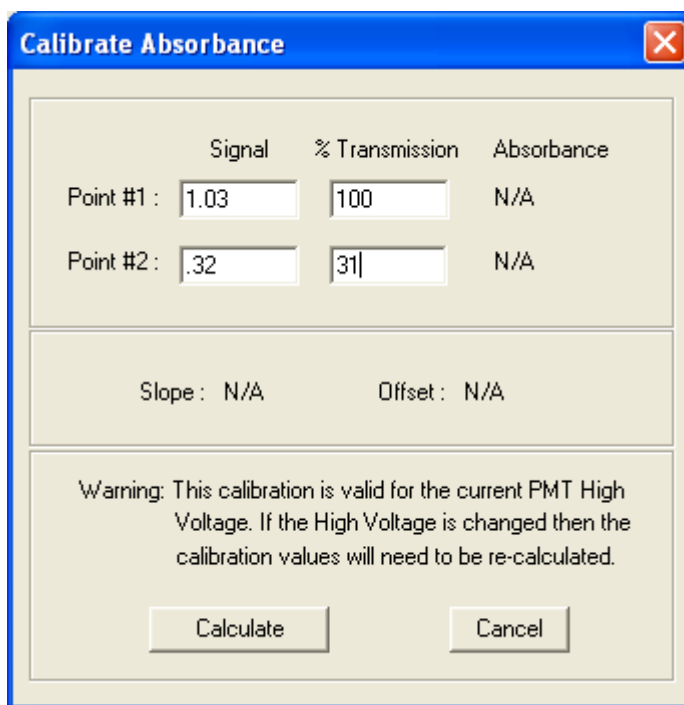
**Figure 2.28:** Convert to Delta Epsilon Panel



routine extinction coefficient used to calculate absorbance. The conversion requires the **molar concentration** of the sample and the cuvette's optical path length (**Cell length**).

The **Convert Intensity to Abs.** math operation converts the CD Current (sometimes called erroneously called the Absorbance signal) to absorbance. This function is only useful for stopped-flow experiments where the PMT current is allowed to change during the experiment. In this case the CD PMT is proportionate to the light transmission through the sample. If this signal is properly calibrated it can be converted to absorbance. A two-point calibration is performed, where the **Signal** and percent transmission (**% Transmission**) from two measurements are entered. The most

**Figure 2.29:** Calibrate Absorbance Panel



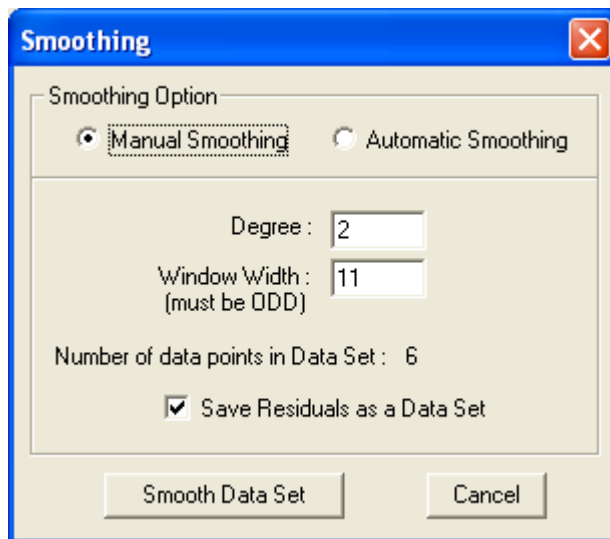
common points for this calibration are 0% and 100% transmission, however, any two signals with known absorbance values can be used. Ideally these points bracket the range of signals seen in the data. Based on these two points the absorbance values are calculated,

as well as the Slope and Offset used to convert the Signal to % Transmission. These values are then used to calculate the absorbance values for the selected data set. For these calculations to be accurate the intensity of light from the source can not change once the

signal levels for the two calibration points has been established. If the CD PMT high voltage level, the slit width, the wavelength, or any other factor affecting the intensity of the source is changed then the calibration will be wrong. For this reason CDS.EXE will automatically lose the calibration values when the CD PMT high voltage is changed.

Smoothing smooths the data using a sliding polynomial. The user may either type in the parameters for the **Manual Smoothing** or use **Automatic Smoothing**, which will find the optimal parameters based on the  $D_2/S_2$  test.

**Figure 2.30:** Smoothing Panel

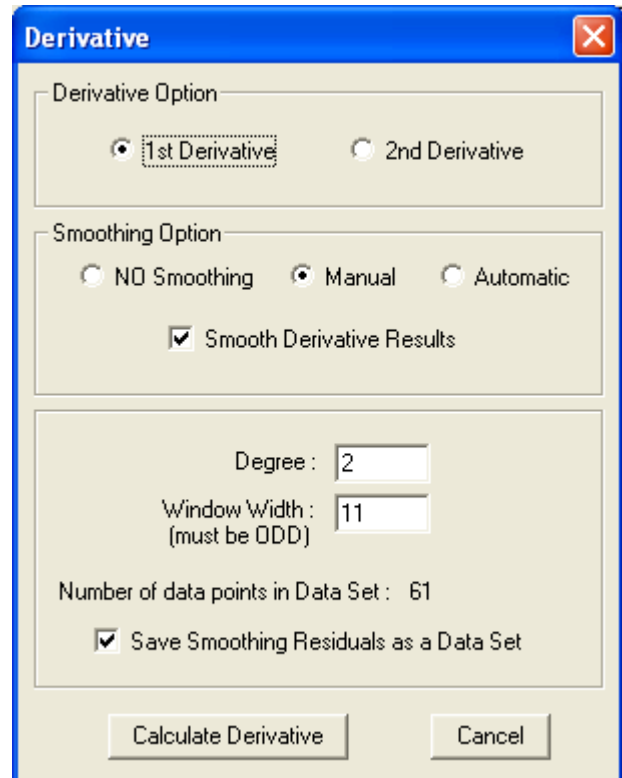


The  $D_2/S_2$  test is a measure of the randomness of the residuals. Random residuals will have a  $D_2/S_2$  value of 2. The **Degree** of polynomial and number of points in the window (**Window Width**) must be entered. The Window Width must be an odd number, so that there are an equal number of points in the window on either side of the data point being smoothed. **Save Residuals as a Data Set** takes the difference between the original data and the smoothed data, and saves it with the name given to the smoothed data plus the word 'residuals'. Smoothing in general does not improve the quality of the data, as it does not

remove noise and can introduce systematic errors. For these reasons all data presented by Aviv instruments is the raw data. Smoothing is left to the discretion of the user.

**Data Set Derivative** takes the difference between adjacent data points to calculate the derivative. Options for the first and second

**Figure 2.31:** Derivative Panel



derivatives are possible. When a derivative is taken high-frequency noise is amplified. For this reason the derivative has options to smooth the signal before the derivative is taken (**NO Smoothing**, **Manual** and **Automatic**), as well as **Smooth the Derivative Results**. The smoothing operations are the same as for the Smooth panel.

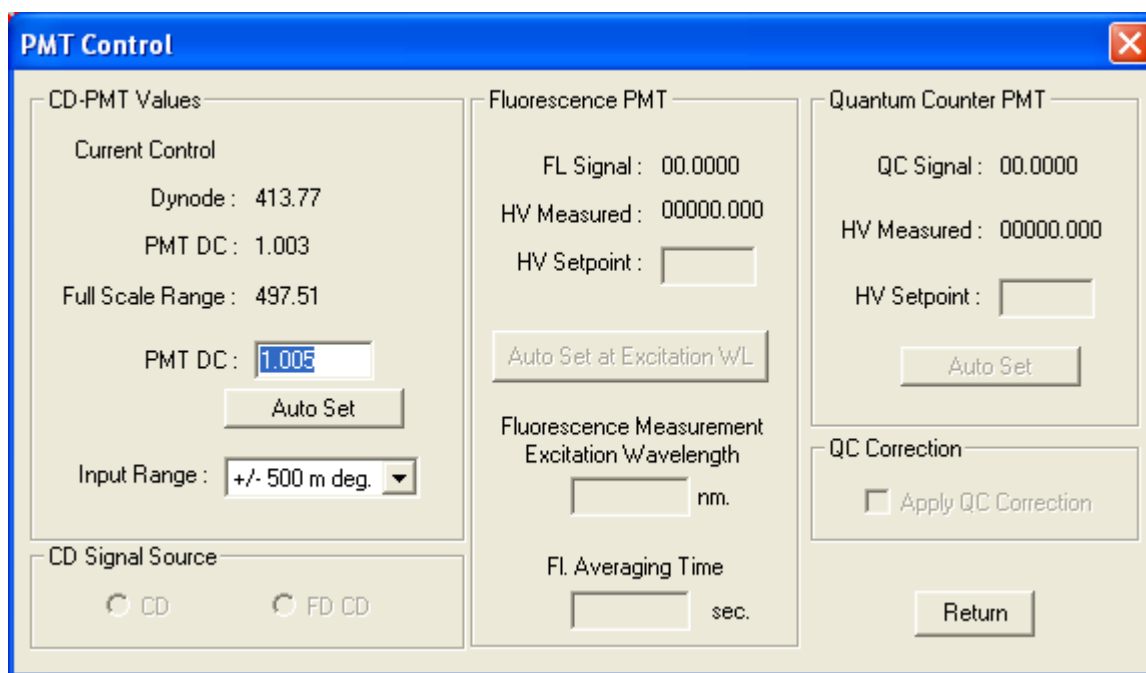
## 2.7 Control Panels

### A. PMT Control, CD

The **PMT Control** panel is where the voltages, and hence the sensitivities of the photomultiplier tubes are control. There are two modes of control for PMTs: constant high voltage or constant current. Constant high voltage control

is mode used for fluorescence and quantum correction measurements. It is also used for stopped-flow CD measurements, where the rate of signal change is too rapid for adjusting the high voltage. By holding the high voltage constant and measuring the PMT current the range of measurement covers roughly 3 magnitudes of light intensity. All of the other

**Figure 2.32:** PMT Control Panel



CD experiment modes use the CD PMT in the constant current control mode. This mode of operation allows the CD instrument to operate over up to 5 magnitudes of light intensity. This range is possible because the gain between the light intensity and output current is roughly logarithmic high voltage. This nonlinearity is not a problem for CD measurements because the CD calculation uses the ratio of the AC ('Delta Absorbance') to DC ('Absorbance') signals from the CD PMT. Both signals contain the same nonlinear gain of the PMT, so the gains drops out when the ratio is taken.

The theory of how the CD signal is measured is describe in "Chapter 1: General Description and Specifications", section "1.2 Theory".

The PMT Control panel is divided into three sections, one section for each possible photo-multiplier. The titles are descriptive of each PMT's function. The displays for **CD-PMT Values** can change depending on whether normal measurements are being make, or the stopped-flow is in use. "Figure 2.32: PMT Control Panel" shows the display for normal operations (stopped flow mode is discussed in "Chapter 9: Stopped-Flow"). **Current Control** indicates that the PMT current is held constant and the high voltage is changed. This mode is used for all experiment modes except stopped-flow. As light intensity goes down the PMT's high voltage (**Dynode** voltage) is automatically raised to increased it's sensitivity. The working

range for this PMT is 170 volts to greater than 500. Values less than 170 result in nonlinear behavior of the PMT due to saturation. Values greater than 500 volts first result in a noisy signal, then the signal dropping to zero as stray light begins to dominate. The loss of CD signal depends on the quality of the optics and sample. **PMT DC** is the set point for the PMT's current. A value of 1 corresponds to 50 microamperes passing through the PMT. **Full Scale Range** is the range of CD signals that can be measured. The range is affected by the PMT DC, where a current of one results in a full scale of  $\pm 500$  millidegrees. Larger PMT DC values reduce the full scale range, and smaller values increase the range. **Auto Set** has no effect in the Current Control mode (it is used for stopped-flow). **Input Range** selects the full scale range that is to be used by the A/D converters. This need not be changed from  $\pm 500$  millidegrees because the increase in sensitivity is not matched by improved electronic noise, which is the limiting factor.

**High Voltage Control** mode (instead of Current Control described before) means that the CD PMT's high voltage is held constant. This mode is used only for Stopped-Flow experiments. This experiment mode has rapidly changing light levels that the high voltage servo can not keep up with, so the high voltage must be held constant. The PMT high voltage must be set for a PMT DC value close to 1 with sample of the correct concentration in the flow cell. This requirement exists because one is the PMT DC level for which the instrument was calibrated. If the Stopped-Flow PMT DC is slightly different (less than  $\pm 0.1$ ) from the value used for wavelength experiments then the error is trivial, but larger differences should be accepted, and the high voltage should be adjusted. If the sample concentration changes (such as from a change in dilution ratio) then the Dynode voltage should be adjusted to bring the PMT DC back to one.

The CD instrument can be used as a single beam spectrometer to measure light absorbance. The method depends on whether the experiment mode is stopped-flow or one of the other experiment modes. In Stopped-Flow mode changes in the PMT DC are proportionate to changes in the Percent Transmission of the sample. This can be calibrated and converted to absorbance, described in "section 2.6 Math Operations", above. Look for the math function "Convert Intensity to Abs." In other experiment modes the high voltage changes with proportionately to the sample's absorbance. Two measurements of high voltage must be made, one of the sample and one of the reference. These two measurements can then be used to calculate the absorbance. The appropriate math function is available through the "Convert Dynode to ABS" function in the Math Operations panel. The QC PMT can be used to improve the quality of absorbance measurements. See below for a description of this accessory.

#### **B. PMT Control, FD CD**

The CD may be equipped with the accessory to measure Fluorescence Detected Circular Dichroism (FD CD). If so then under the heading of **CD Signal Source** are buttons for **CD** and **FD CD** are enabled. Normally the instrument measures the CD signal. If the accessory is available then the CD signal can be measured through the fluorescence photomultiplier tube (Fluorescence Detected CD). This accessory uses the fluorescence PMT to detect the CD signal, so the Fluorescence PMT must first be properly set. The recorded signal is the difference in intensity between the fluorescence signal resulting from excitation from left and right fluorescence light. Sometimes this signal is published as divided by the total fluorescence, so the total fluorescence value via the PMT DC signal should also be recorded for future use.



### C. PMT Control, Fluorescence

The **Fluorescence PMT** section of the PMT Control panel contains the controls for fluorescence measurements. These controls are only available if the software detects this accessory at startup. **FL Signal** and **HV Measured** are the current values for these parameters. The **HV Setpoint** allows the user to manually control the high voltage applied to the fluorescence PMT, which in turn controls the sensitivity of the device. The full scale is 0 to 5, with 1 being the typical value. Larger high voltages increase the sensitivity, but may saturate the PMT if the light level is too high. The high voltage working range is 170 to 1000 volts. The **Auto Set** button automatically raises the high voltage to achieve a signal level of 1. This feature should be used after the **Fluorescence Measurement Wavelength** is set. This determines the excitation wavelength that will be used for fluorescence measurements if both CD and fluorescence measurements are made during an experiment. The emission wavelength is determined by the glass filter placed in front of the fluorescence PMT (see "Chapter 7: Thermoelectric Cell Holders" for more information). The instrument will automatically toggle back and forth between the CD and fluorescence excitation wavelengths to make measurements over the course of the experiment. **FI Averaging Time** is the time the fluorescence signal will be averaged to generate a data point.

### D. PMT Control, Quantum Counter

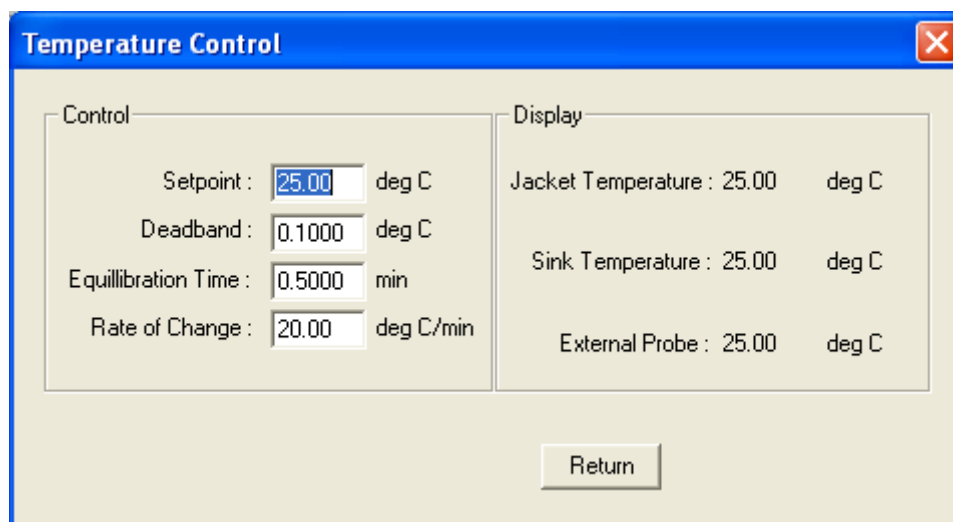
The **Quantum Count PMT** is another accessory that is used to measure the light intensity output of the instrument. This signal can be used to correct absorbance and fluorescence signals. The CD instrument is fundamentally a single beam instrument, with no reference beam. The CD signal is self referencing, so it does not need this accessory (see above for explanation). The absorbance and fluorescence signals will change as a function of the light output of the instrument. To remove this

unwanted influence these signals can be ratioed with the QC signal. **QC Signal**, **HV Measured**, **HV Setpoint**, and **Auto Set** all operate the same way as described above for the Fluorescence PMT. If the **QC Correction** button is turned OFF then the saved fluorescence and absorbance signals will be the raw signals. If this option is turned ON then these signals will automatically be ratioed with the QC Signal and the corrected signals will be saved.

### E. Temperature Control

The **Temperature Control** panel sets the temperature of the thermoelectric cell holder. There are several versions of this accessory, but they all fundamentally work the same. They use Peltier elements that act as heat pumps. When an electrical current is passed through a Peltier element one surface becomes hot and the other cold. The temperature of the cell holder is controlled by adjusting the current through the Peltier elements. Temperature sensors in the cell holder jacket measure the temperature, and the control box then uses this information to calculate the appropriate amount of heat to add or remove to the cell holder. The temperature controller has a dedicated microprocessor to do the decision making, and to communicate with the computer. When the controller is first turned on it initializes itself for the first several seconds. At this time it determines the type of thermoelectric cell holder that it is controlling. This information is passed to the computer, that then selects the appropriate user interfaces, tuning parameters and calibration constants. The user does not have to make any software changes when cell holders are changed. A chiller set at 20C is used to remove waste heat from the heat sinks on the Peltier elements. The chiller temperature should not be changed.

The left side of the Temperature Control panel contains the **Control** settings. **Setpoint** is the temperature set point. When this value is

**Figure 2.33:** Temperature Control Panel

changed the temperature controller automatically drives the temperature to the new set point. **Deadband** is the allowable error in the temperature before measurements are made. If the difference between the set point and the device's temperature is greater than Deadband then measurements will be delayed. The **Equilibration Time** is the time between when the measured temperature first arrives within the deadband, and when the optical measurements are started. This time allows thermal gradients to dissipate and the whole system reach the same temperature. **Rate of Change** is the rate at which a thermoelectric will approach the Setpoint. This is useful for controlling the amount of temperature overshoot when adjusting the temperature.

On the right side of the Temperature Control Panel are the measured temperatures. This display is different for the different types of thermoelectric cell holders. In all cases the top temperature is the temperature used for the feedback loop (usually **Jacket Temperature**). The **Sink Temperature** is the temperature of the water sink used to remove waste heat from the system. This temperature is normally a degree or two above the temperature of the incoming cooling water. During changes in the

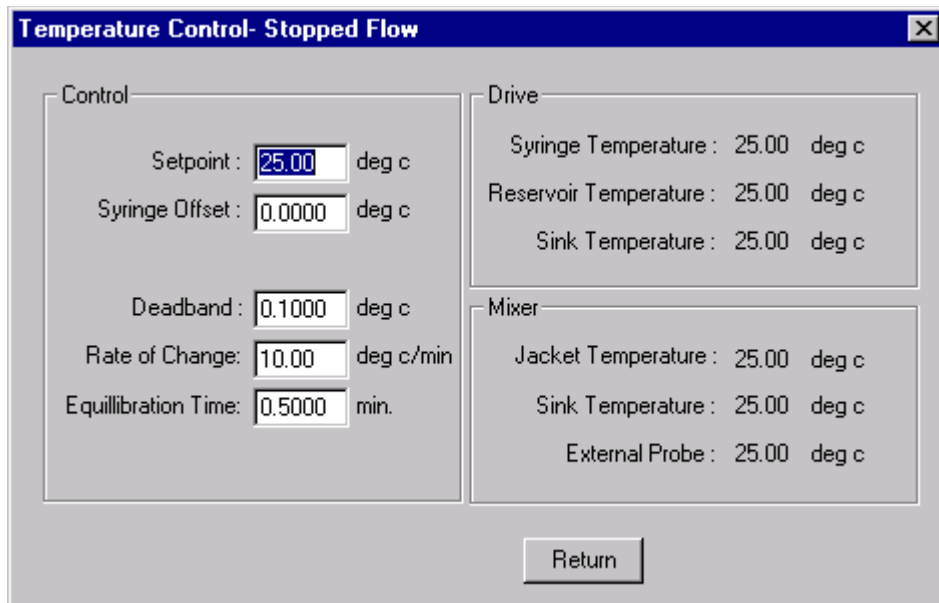
thermoelectric cell holder's temperature the Sink temperature may deviate, but will return once the cell holder has equilibrated. If there is insufficient cooling (such as the water is not flowing) then the sink temperature will rise. If the Sink Temperature reaches approximately 38 C then power to the Peltier elements is turned off, and temperature control will be lost. When the sink temperature cools the power will automatically come back on. An **External Temperature** probe is available as an accessory. This probe sits in the sample compartment, and can be located where ever desired.

If the cell holder is a Stopped-Flow accessory then a second control loop is used for the syringes and reservoir. Normally this loop is held at the same temperature as the Mixer. However **Syringe Offset** can be used to create a different temperature. The temperature readings are divided into the regions the two loops control: the syringe **Drive** and **Mixer**.

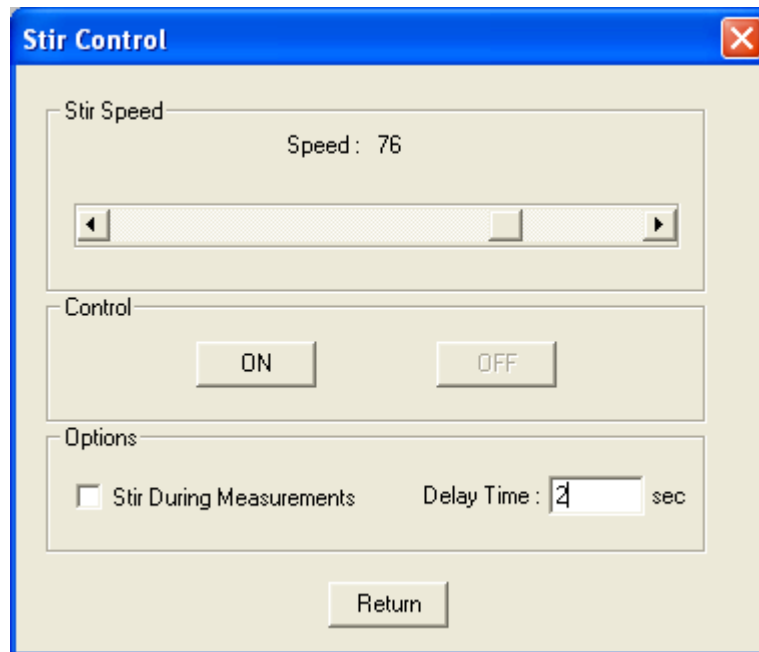
#### F. Stir Control

Most Aviv thermoelectric cell holders come equipped with a motor attached to a magnet beneath the cuvette. This mechanism is used

**Figure 2.34:** Temperature Control Panel, Stopped-Flow



**Figure 2.35:** Stir Control Panel



to drive magnetic stir bars placed in samples. The speed of the motor is controlled via the temperature controller, which receives its instructions from the computer. The **Stir Control** panel is used to turn the stirring **ON**

and **OFF**, as well as set the stirring **Speed** with the slide bar. The Speed units are arbitrary. During Titration experiments the maximum Speed is recommended. Under **Options** is **Stir During Measurements**. If this option is turned

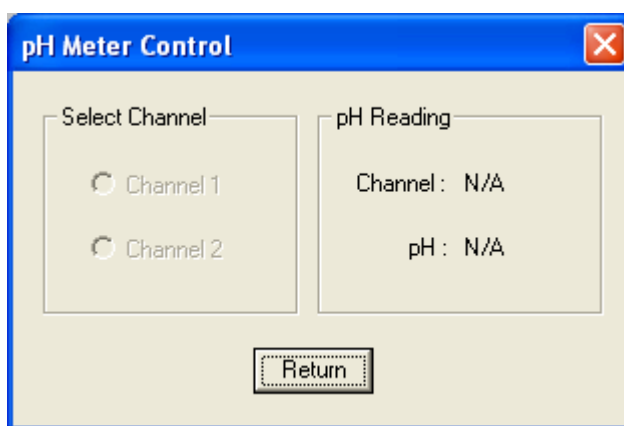
ON then the stirrer will continue to stir during optical measurements. Of this is turned OFF then stirring is stopped prior to optical measurements, then resumed when measurements are finished. When the stirring is stopped the software waits the **Delay Time** before making optical measurements. The advantage of not stirring during measurements is that if there are bubbles or dust in the

sample then not stirring give a chance for these to move out of the light beam, thus improving the measurement quality.

### G. pH Meter Control

If the instrument is equipped with the pH meter accessory then this device can be communicated with through the **pH Meter Control** panel. The meter has two channels who's readings can be selected. Channel 1 is used for all experiments.

**Figure 2.36:** pH Control Panel



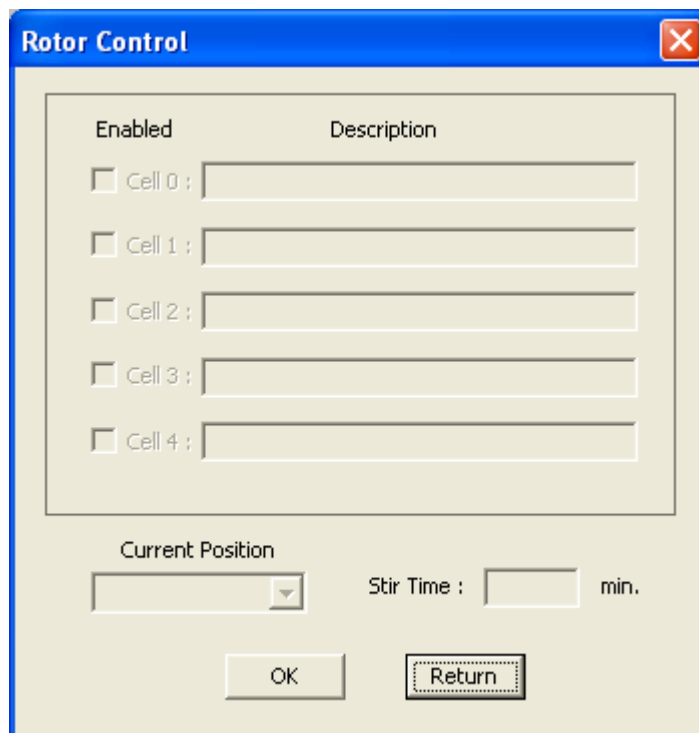
### H. Rotor Control

The **Rotor Control** panel controls the actions of the five position cell holder. If this accessory is detached then the options are greyed out. To **Enable** measurements on select positions during experiments turn on the check boxes for the selected cells. Each position can have its own **Description** that will be saved with the Data file. The cell selected for the **Current Position** is the one in the light path and being measured, and can be manually set. Beneath this position is a magnet attached to a motor, used for driving magnetic stir bars. A **Stir Time** can be set to delay measurements after the sample has moved into the measuring position, to allow the sample to be stirred before optical measurements are made.

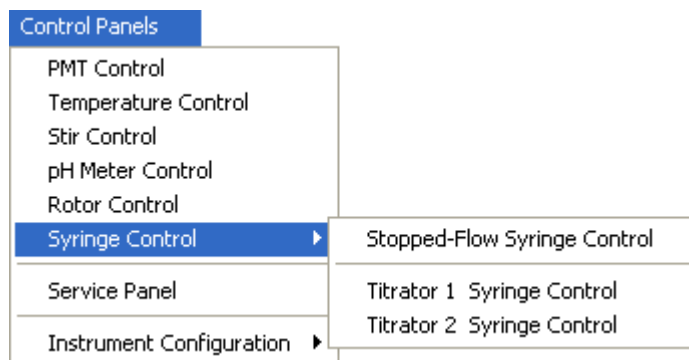
### I. Syringe Control, Stopped Flow

The **Syringe Control** menu of the Control Panels menu has selections for the **Stopped-Flow Syringe Control**, **Titration 1 Syringe Control** and **Titration 2 Syringe Control** (Figure 2.38: Syringe Control Submenus). The explanations of the software given here are not complete instructions for the operation of the stopped-flow. Consult "Chapter 9: Stopped-Flow" for additional instructions. The **Syringe Control** panel for the Stopped-Flow is divided into four sections, one for each syringe (Figure 2.39: Stopped-Flow Syringe Control Panel). Each section graphically shows the syringes' volume, with the **Current Volume** shown below. When this panel is opened for the first time the syringes have not been homed and the volumes are not known, and are not shown. The Current Volume is based on the actual read-back from the motor's optical

**Figure 2.37:** Rotor Control Panel



**Figure 2.38:** Syringe Control Submenus



encodes, so small variations may occur. The **Syringe Size** is the maximum volume for the syringe. **Set Volume** is the volume that the plunger will move to when commanded. Below are the **Syringe Descriptions**, where the user can type in the contents of each syringe. This information is displayed in the Stopped-Flow experiment configuration panel and is saved to the data file. After a description is typed in the Enter key should be hit to save the text changes.

In the Stopped-Flow Syringe Control panel the **Exit** menu item closes the panel. Menu items **Syringe 1** through **Syringe 4** contain identical menus, except they apply to different syringes. If the physical syringe has been recently changed, then **Change Syringe Size** may need to be updated. As a reminded, this chapter has only descriptions of the software. Details of the operation are contained in Chapter 9: Stopped-Flow. Before any motions are possible with the syringe positions must be

Figure 2.39: Stopped-Flow Syringe Control Panel

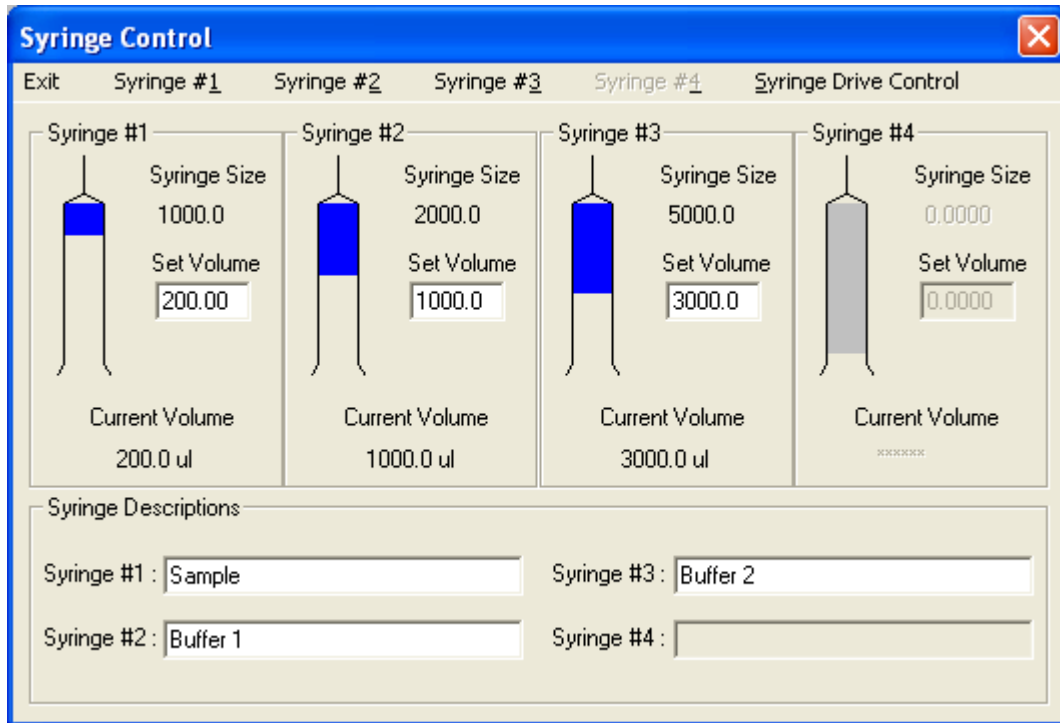
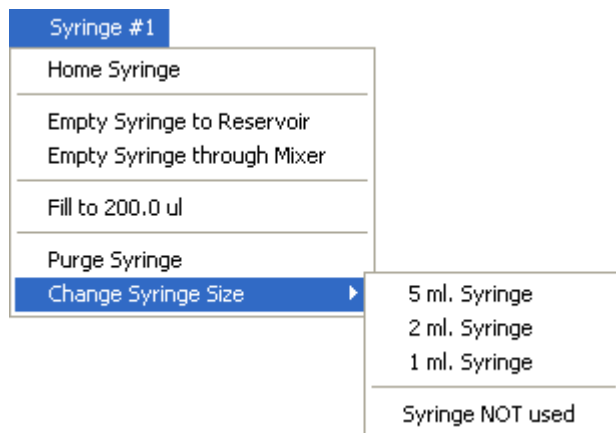


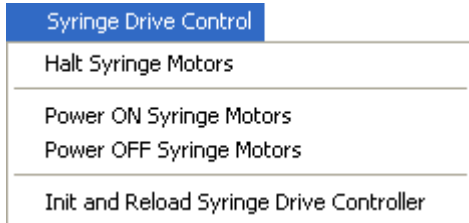
Figure 2.40: Stopped-Flow Syringe Control Menu



homed. **Home Syringe** moves the plunger through the range of motions so the that hardware and software knows the limits. After this operation the other motion commands become operable. **Empty Syringe** moves the plunger to the zero volume position. Although the software may give options for destination of the

fluid, it is the responsibility of the user to move the values to the appropriate positions. If the volume is less than that Set Volume, then the option to the syringe becomes available. **Fill Syringe** moves the plunger to the absolute volume that is entered in Set Volume. If the Current Volume is more than the Set Volume then the option changes to **Empty to the set volume**. Therefore Fill Syringe can be used to partially empty the syringe as well. **Purge Syringe** is used for removing air bubbles from the syringe. It will move the plunger to approximately 25% of the syringe then quickly empty. The command should be repeated until no bubbles are seen in tubing or reservoir. There are menus for each of the syringes, and the syringes can be operated simultaneously and independently of each other. The **Syringe Drive Control** menu contains items not frequently used. **Halt Syringe Motors** will stop the motor movement immediately. However, a faster way to stop the motors is to use the Emergency Stop button on the front of the

**Figure 2.41:** Syringe Drive Menu



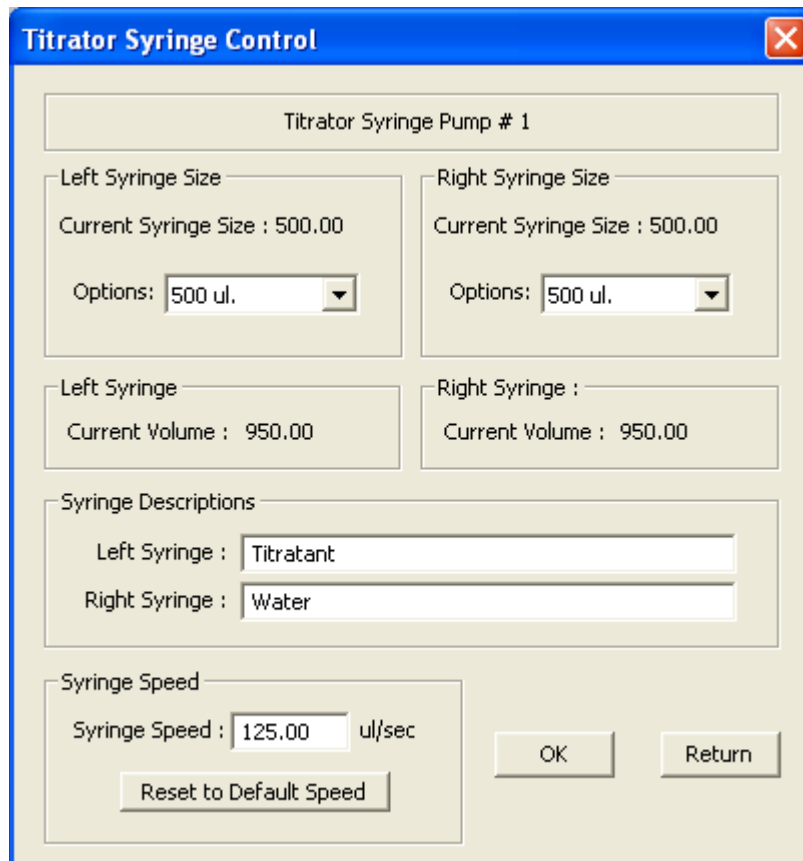
stopped-flow syringe power supply. **Power ON Syringe Motors** and **Power OFF Syringe Motors** is used to turn the power on and off to the motors. Normally this is done automatically. If it is necessary to work on the syringes then the motor power must be off so the plungers can be moved up and down by hand. As a safety precaution it is advised that the power switch to the stopped-flow motor power supply

be turned off while the syringes are being worked on. When work is completed the switch power can be turned back, then the **Power ON Syringe Motors** command given to send power to the motors. It is recommended to **Home** the motors again after servicing them. **Init and Reload Syringe Drive Controller** will clear the program on the motor controller card and load the program fresh into it's memory. This should only be used if the motion control or position has become confused, and a fresh start is necessary. This will clear the syringe positions, so it will be necessary to repeat the Home function for each syringe.

**J. Syringe Control, Titrator 1 Syringe Control and Titrator 2 Syringe Control**

These are used to control the optional syringe pumps. The menus for both pumps are iden-

**Figure 2.42:** Syringe Drive Menu



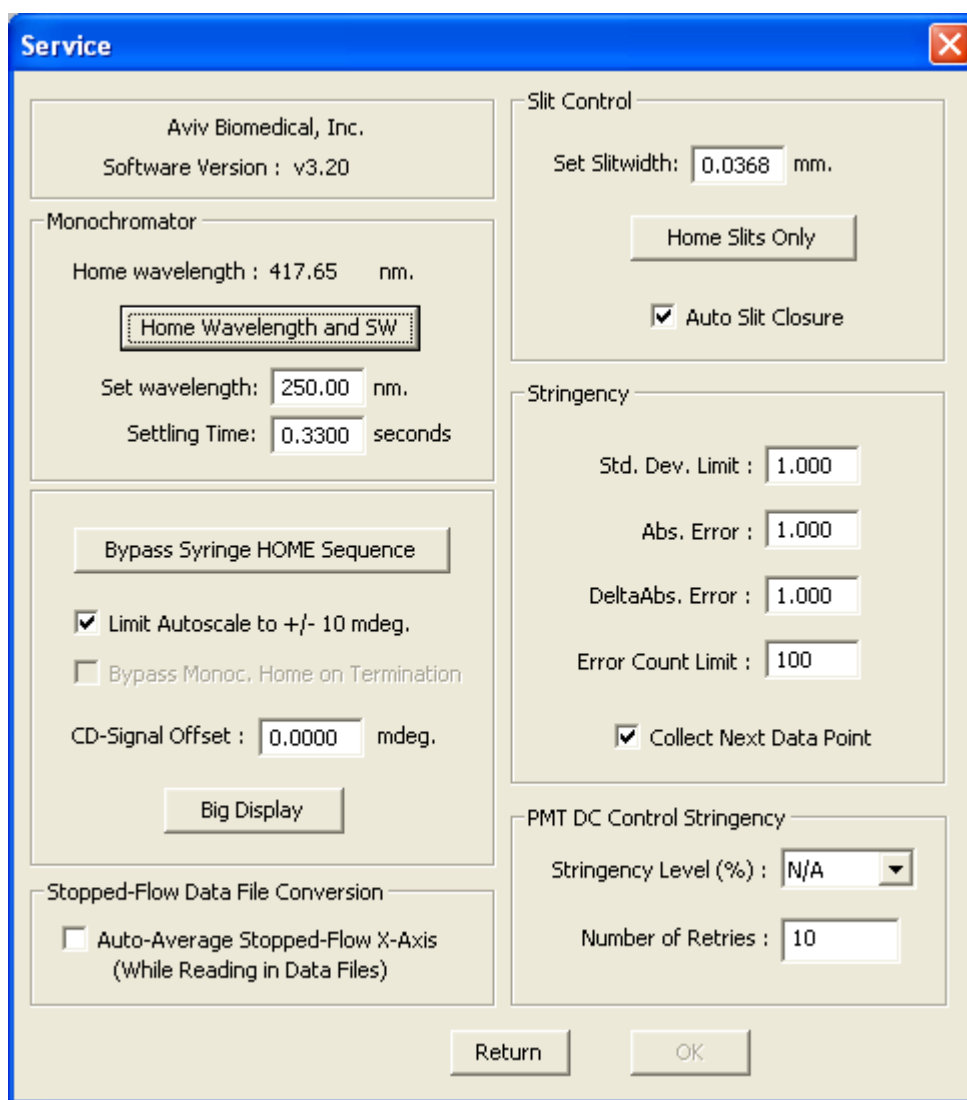
tical so only one explanation is necessary. The Titration and pH experiments modes use only Titration Syringe Pump 1. The Macro experiment language can control both pumps. For the software to properly calculate the injection volumes the **Left Syringe Size** and **Right Syringe Size** must both be properly selected from the lists of possible sizes. The **Current Volume** for both syringes are read back from the pumps and displayed. **Syringe Descriptions** can be entered, and this information will

be saved as part of the data files. The Enter key must be used at the end of the description. The **Syringe Speed** can be adjusted if a speed other than the default gives better performance. **Reset to Default Speed** sets the speed back to 125 ul/sec.

#### K. Service Panel

The Service Panel contains controls that are not routinely needed by users. There are

Figure 2.43: Syringe Drive Menu





some useful features that are worth mentioning. The top left corner has the **Software Version**. The **Home Wavelength and SW** button will send the wavelength and slit width drives to their home positions. **Home Slits Only** affects only that piece of hardware. The **Big Display** button opens a display panel showing the current PMT signals and voltages in large letters. This feature can be helpful when aligning the lamp or doing other service work in awkward positions. The **OK** button is greyed out to avoid values being changed. These parameters can have severe effects on the instrument performance and should only be adjusted with Aviv Biomedical supervision.

#### **L. Instrument Configuration**

The items under this menu item are all related to the functioning of the hardware and not to routine use. **Print Configuration** sends a list of the current settings to the LPT port of the computer, so that a paper copy of the settings can be saved.

## Chapter 3: Sample Preparation for CD Measurements

### 3.1 Absorption of Light

When preparing a sample for CD measurements the absorption of light must be considered. For normal light the optical density (OD) of the sample is given by the Beer-Lambert equation:  $OD = \epsilon \cdot L \cdot c$ , where  $\epsilon$  is the extinction coefficient (OD/cm\*Molar),  $L$  the path length (cm) and  $c$  the sample concentration (Molar). CD is based on the absorption of left and right circularly polarized light, so the Beer-Lambert equation still applies. However, instead of the extinction coefficient, the difference in the extinction coefficients for left and right circularly polarized light is used ( $\Delta\epsilon = \epsilon_L - \epsilon_R$ ). Therefore the CD signal linearly tracks the sample concentration. From this relationship one would naturally assume that higher sample concentrations would give better CD signals. Unfortunately this case is not true. The  $\Delta\epsilon$  is small compared to the absolute levels of light. Increasing the concentration results in less light for the measurement, and the small difference in absorbance can become lost in the noise and stray light. Remember that absorbance is the log scale of transmission, which is the true measure of the light energy. An absorbance of 1 means that 90% of the light is being absorbed, 2 means 99%, 3 equals 99.9%, and so on. The noise level is not constant but is also a function of the light level, being higher at low light levels. Therefore the signal-to-noise profile peaks at approximately 0.8 OD before deteriorating. The profile is relatively broad so deviations are acceptable. However, it is always desirable to have an absorption level in the range of 0.8 OD at the wavelength of interest.

tion will scale the absorbance linearly. The researcher can adjust concentrations and path

### 3.2 Solvent Transparency

The researcher must consider what is actually absorbing the light. The ideal situation is for the sample molecules and nothing else to be the active chromophores. Unfortunately, in the UV wavelength range, and particularly the deep UV (below 250 nm), many things absorb light. This effect only reduces the amount of light available for the measurement and adds nothing to the CD signal. When taken to the extreme there is so little light passing through the sample that CD measurements are impossible. This is frequently the case when taking wavelength scans of proteins and it is desirable to penetrate the UV as far as possible. Table 3.1: Solvent Transparency shows the wavelengths at which a given solvent has an OD of 1. Values are given for optical path lengths of 1.0 and 0.05 mm. For a given solvent and path length wavelengths longer than the ones in the table will have lower absorbances while shorter wavelengths result in rapidly increasing absorbances. The problem of solvent absorbance can be reduced by using short path length cuvettes and scaling up the sample concentration. For example, a 0.5 mm cuvette with 2X the sample concentration will have the same CD signal as a 1X sample concentration in a 1 mm cuvette, but with 1/2 the solvent absorbance.

### 3.3 Salt and Buffer Selection

The same absorbance problems occur with buffers and salts added to the sample. Table 3.2: Absorbance of Various Salt and Buffer Substances in the Far-UV Region shows the absorbance values for different compounds over a range of wavelengths. The conditions are for a 1.0 mm cuvette containing 10 mM solutions. Changing path length or concentration to optimize CD measurements.

**Table 3.1: Solvent Transparency**

Compound	Wavelength (nm) for OD = 1.0	
	1.0-mm Path Length	0.05-mm Path Length
H <sub>2</sub> O	182	176
MeOH	195.5	184
F <sub>6</sub> iPrOH	174.5	163
F <sub>3</sub> EtOH	179.5	170
EtOH	195	186
MeCN	185	175
Dioxane	231	202.5
Cyclohexane	180	175
$\alpha$ -Pentane	172	168

**Table 3.2: Absorbance of Various Salt and Buffer Substances in the Far-UV Region**

Compound	pH	No Absorbance Above	Absorbance of a 10 mM solution in a 1.0 mm Cuvette at:			
			210 nm	200 nm	190 nm	180 nm
NaClO <sub>4</sub>		170 nm	0	0	0	0
NaF, KF		170 nm	0	0	0	0
Boric Acid		180 nm	0	0	0	0
NaCl		205 nm	0	0.02	>0.5	>0.5
Na <sub>2</sub> HPO <sub>4</sub>		210 nm	0	0.05	0.3	>0.5
NaH <sub>2</sub> PO <sub>4</sub>		195 nm	0	0	0.01	0.15
Na Acetate		220 nm	0.03	0.17	>0.5	>0.5
Glycine		220 nm	0.03	0.1	>0.5	>0.5
Diethylamine		240 nm	0.4	>0.5	>0.5	>0.5
NaOH	pH 12	230 nm	>0.5	>2	>2	>2
Boric Acid, NaOH	pH 9.1	200 nm	0	0	0.09	0.3
Tricine	pH 8.5	230 nm	0.22	0.44	>0.5	>0.5
TRIS	pH 8.0	220 nm	0.02	0.13	0.24	>0.5

**Table 3.2: Absorbance of Various Salt and Buffer Substances in the Far-UV Region**

Compound	pH	No Absorbance Above	Absorbance of a 10 mM solution in a 1.0 mm Cuvette at:			
			210 nm	200 nm	190 nm	180 nm
HEPES	pH 7.5	230 nm	0.37	0.5	>0.5	>0.5
PIPES	pH 7.0	230 nm	0.2	0.49	0.29	>0.5
MOPS	pH 7.0	230 nm	0.1	0.34	0.28	>0.5
MES	pH 6.0	230 nm	0.07	0.29	0.29	>0.5
Cacodylate	pH 6.0	210 nm	0.01	0.20	0.22	

### 3.4 Cuvette Selection

Selection of the appropriate cuvette is also important. Cuvettes are available from a wide range of materials, few of which are suitable for CD measurements. First, the cuvette should be capable of transmitting light in the range of interest. Table 3 shows the approxi-

mate wavelength range for which the light transmission is better than 80%. For CD measurements in the deep UV the best material is UV grade fused silica. At wavelengths below 200 nm all commonly available cuvettes absorb light, so measurements become difficult regardless of the sample characteristics.

**Table 3.3: Selection of Cuvette**

Material	Transmission >80%
UV grade fused silica	200 - 2500 nm
Fused silica	230 - 2500 nm
IR grade fused silica	200 - 3500 nm
High quality optical glass"	320 - 2500 nm
Optical glass"	360 - 2500 nm
Pyrex	340 - 2500 nm
Methacrylate Plastic	300 - 750 nm
Polystyrene Plastic	350 - 750 nm

Second, the cuvette should not be mechanically strained. Strain in the cuvette will depolarize the light so that the CD baseline is not flat. When buying cuvettes it is possible to specify "Strain Free" cuvettes from the manufacturer, but this specification is not always sufficient. The manufacturer screens the cuvettes in the visible range then sells the best

as strain free. Unfortunately strain becomes more apparent at shorter wavelengths, and even a "Strain Free" cuvette could have a poor CD baseline. Buying cuvettes from Aviv eliminates the risk of receiving an unsuitable cuvette.

Cuvettes are available in path lengths from 0.001 cm to 10.00 cm. The 0.1 to 0.001 cm range of cuvettes are composed of two parts for easy cleaning. Long path length cuvettes (>2 cm) should be used only when absolutely required, due to the possibility of artifacts caused by scattering. Rectangular cuvettes less than 1 cm should have spacers to provide accurate positioning in the holder. Contact Aviv for a full description of the available cuvettes and spacers.

The least expensive solution to obtain a "Strain Free" cuvette is to screen all available cuvettes in the laboratory, and to set aside the suitable ones as being used for "CD Only". Cuvettes are tested by comparing the cuvettes' water baseline to the instrument air (no cuvette) baseline. Significant deviations between these two (>5 millidegrees) eliminates a cuvette. The cuvette baseline should show no peaks (either positive or negative). The cuvette baselines should be taken in all possible orientations.

### 3.5 Sample Preparation

Samples should be homogeneous and filtered (if necessary) to remove particulate matter, which can cause errors due to light scattering. If filtering is undesirable then briefly centrifuge the sample to remove particulate matter. Avoid lint by using lens paper instead of laboratory tissue (Kimwipes, for example). If temperature or titration experiments are to be done then the sample should be immediately before placing it in the CD. Changes in temperature or solvent conditions reduces the solubility of atmospheric gasses in your sample. This effect will result in the sample outgassing, causing in bubbles in the light path. A bubble in the light path can cause large CD signal deviations. If the cuvette is 1 cm then stirring is recommended to reduce the size and effect of the bubbles, as well as photobleaching.

### 3.6 Importance of the Dynode Voltage

The light is measured using a device called a Photomultiplier Tube (PMT). This device is unique among light transducers in that its sensitivity can be adjusted. By applying increasing high voltages to the PMT's dynodes the sensitivity the PMT is increased. CD spectrometers are unusual (compared to fluorometers, for example) in that CDs vary the dynode voltage while maintaining a constant DC current from the PMT to measure the CD signal. As a result the CD is capable of operating over a large range of light intensities. Unfortunately the raw CD signal is somewhat weak, so a minimum amount of light is necessary to make the measurement. Therefore you will see a correlation between the light level, dynode voltage and the quality of the CD measurement. "Good" light levels are correspond to dynode voltages approximately 200 Volts and low noise. CD noise increases with the dynode voltage, to roughly 500 volts ("low" light), where measurements become fade out. A dynode voltage that plateaus at an elevated level is an indication that only stray light is being measured and the CD signal should not be trusted. In this situation there is not only random noise, but also the measured CD signal is weaker than the real CD signal.

### 3.7 Photobleaching

In some cases the light energy is sufficient to alter the chemistry of the sample molecule. This can happen even in samples that have no known photochemistry or no absorption in the visible wavelengths. Evidence of photobleaching is drift in the CD signal over time. Sometimes this drift can not be recognized because over the time course of a single experiment. The test of photostability is to perform kinetic measurements of same sample over the same time, light and buffer conditions as the other experiments, but keeping other variables like temperature or concentration constant. Evidence of photobleaching is an exponential decay in the CD signal.

Photobleaching occurs as a function of the number of photons (light flux), which can be controlled by changing the slits width. The slit width is determined by the bandwidth setting. Halving the slit width / bandwidth will reduce the light by approximately four fold, resulting in nearly a four fold reduction of the photobleaching rate. If the kinetics show no sensitivity to the light intensity then they are not due to photobleaching. This is the classic test to distinguish between other possible kinetic sources.

Stirring can help reduce photobleaching. If the sample is not stirred then the light will “burn a hole” through the sample, resulting in a column of bleached molecules where the light is present. Stirring will refresh the bleached molecules with fresh ones, reducing the apparent rate of photobleaching and its kinetics. Stirring is not possible in cuvettes <1 cm.

### 3.8 Baseline Correction

Experiments that require absolute measurement of the CD signal require subtraction of the baseline. This requirement is particularly true when doing secondary structure estimates. Measurements of relative changes in the CD signal (temperature melts or solvent statutorily, for example) do not require this correction. Effort should be taken to match the baseline measurement conditions to the sample conditions as well as possible. Baseline spectra should be measured using the same experiment conditions and the same cuvette in the same orientation in the light path with the same buffer system at nearly the same time as the actual experiment. The baseline can be subtracted using the **Math Operations, Subtract Data Sets** operation in the CD instrument software. Select the sample CD signal as **Data Set A** and the buffer CD signal as **Data Set B**. The result will be saved in the sample's data folder.

### 3.9 Normalization of Data

Some forms of data analysis require the CD signal be normalized for concentration and sample path length. For many samples the **Convert to Delta Epsilon** math function will work. For proteins however, it is necessary to normalize the CD signal for the amino acid concentration, and a different sample path length (decimeters instead of centimeters). These steps are done with the **Convert to Molar Ellipticity** operation. The normalized data set can be saved to a text file for analysis highlighting it in the **Data Browser** and using the menu options **Export Data Set: Export to ASCII Text**.

### 3.10 Care and Cleaning of Cuvettes

Cuvettes should not be touched on the optical windows. Highly alkaline solutions should not be left in them for extended periods. Solvents with a pH greater than 10 can etch the glass. A cuvette should be rinsed and soaked in distilled water immediately after use. Clean with a mild detergent solution containing no lanolin, oils, or other contaminants. Some detergents are alkaline (Alconox, for example), so check the pH before soaking the cuvette in a detergent. Proteins may be removed by soaking in a mixture of equal parts of 3 N HCl solution and 50% ethanol. Overnight soaking in a mild pepsin solution may be helpful at times. After cleaning, cuvettes should be rinsed in distilled water and gently blown dry with clean, dry air or nitrogen.

## Chapter 4: Maintenance

### 4.1 Introduction

Your Aviv 410 Circular Dichroism Spectrometer has been designed to be generally trouble free. Routine maintenance such as changing the lamp, checking the calibration, and instrument energy levels are described here. Certain simple tests should be periodically performed to insure that the instrument is operating properly. These tests should be performed at least every time the lamp is changed, if not more frequently.

This section assumes the user is familiar with the CD software. You should consult the other chapters if you have any questions, or call Aviv Biomedical for support.

### 4.2 Changing the Xenon Lamp

#### Warnings:

**SAFETY GLASSES & GLOVES must be worn.**

Follow the manufacturer's safety warnings on the replacement lamp.

Make sure the power has been off for at least 20 minutes.

The lamp must be cool prior to handling.

If the lamp has a finger print or other dirt it will bake onto the glass, creating a hot spot and possible breaking the glass. Clean the lamp with a fresh tissue and ethanol.

The lamp housing is located on the back, right corner of the instrument, when facing from the front.

1) **Turn the instrument and lamp power off.**

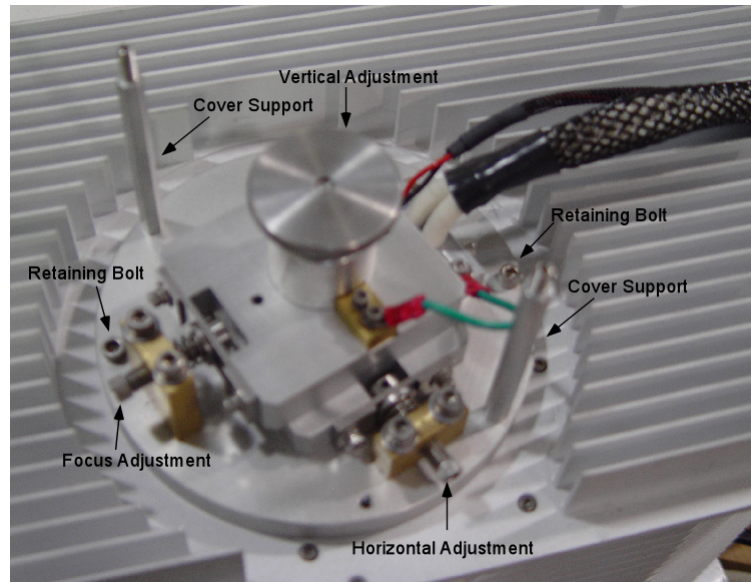
2) Remove the thumb nuts on the top of the white round lamp cover (see Figure 4.1: Lamp Cover) and lift the cover upwards and off.



**Figure 4.1:** Lamp Cover

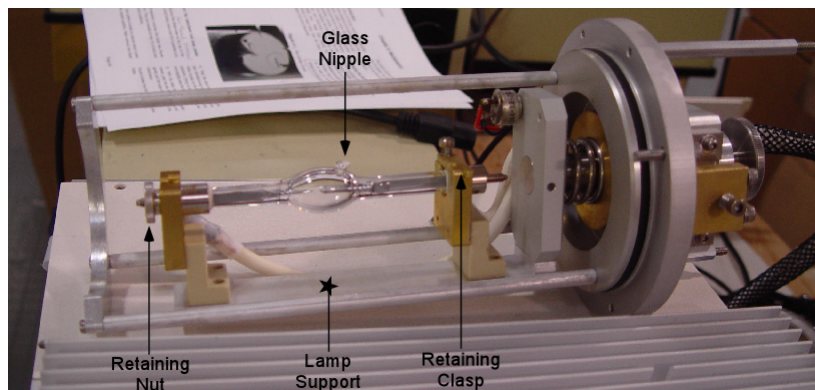
3) Completely unscrew the back two Retaining Bolts. They are located at the front and back positions. Use a 9/64" hex wrench to remove the bolts.

4) Grip the two Cover Support posts and lift up. It may be necessary to gently rock side-to-side to break the seal. Slowly and gently pull the lamp holder up and out of the instrument.



**Figure 4.2:** Lamp Mechanism

5) Lay the lamp holder on its side beside on top of the instrument, beside the lamp compartment. Remove the retaining nut.



**Figure 4.3:** Lamp Connections

6) Lift the Retaining Clasp so the top post of the old lamp can be lifted out of the top support. Pull the lamp post free of the bottom support. Set the old lamp aside.

7) Do not remove the protective cover from the new lamp yet. The top of the lamp support has a "+" etched into it. The lamp has a "+" etched into on a post as well. Place the unmarked post into the bottom of the support, and lay the "+" into the notch at the top. The Glass Nipple of the lamp must either face the

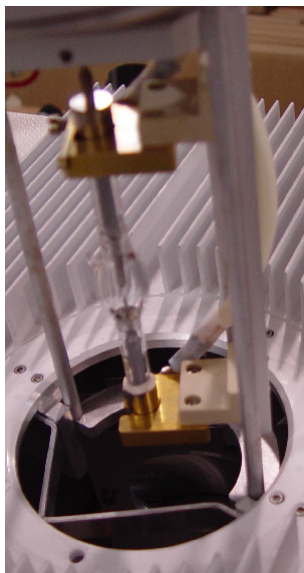
Lamp Support, or face directly away from it. The light path is through the open area, and the nipple must not interfere with the light.

8) Fasten the Retaining Nut back onto the exposed lamp bottom post. It should be snug with finger strength, not hard. Swivel the Retaining Clasp over the top lamp post.

9) Remove the protective cover from around the new lamp, and place the cover on the old lamp.



10 Place the lamp housing back into the instrument. There is only one position that the housing can fit into the compartment. Firmly press the housing down to seat the seal.



**Figure 4.4:** Lamp House Position

11) Replace the two Retaining Bolts to secure the lamp house.

### 4.3 Adjusting the Xenon Lamp

A) Ignite lamp and leave on for at least seven hours.

B) Start up the instrument and software.

C) The Main Control panel will appear. Monochromator settings will be displayed towards the bottom left of the screen. Double click on wavelength, enter 200 and hit return.

D) Single click on **Control Panels** (upper right), single click on **Service Panel**. In the Service Panel click on the **Big Display** button (lower left quadrant of panel). A panel will open that displays the dynode voltage and signal in large, high contrast characters.

E) Turn the monitor towards the lamp house. Select a comfortable position by the lamp house to make adjustments. Make sure you can see the dynode voltage on the monitor while making lamp adjustments.

F) The largest difference between lamps is the height of the arc, so this adjustment is expected to produce the greatest improvement. The **Vertical Adjustment** knob is the large one in the center of the lam house.

Slowly turn this knob up or down to obtain the lowest dynode voltage (most light) possible.

G) Next gently adjust the **Horizontal Adjustment** with a 9/64" hex wrench. Small movements can cause large light changes, so be careful.

H) Repeat the prior two steps several times until further decrease in the dynode voltage is possible. Do not adjust the Focus Adjustment as this will have little effect on the light intensity. The voltage should be below 250 volts. If this goal is not reached then call Aviv Biomedical for further assistance.

### 4.4 Wavelength Calibration

The NIST 2034 holmium oxide standard is useful in calibrating the wavelength of the monochromator. The wavelength drive is very precise, and coupled with the very narrow holmium oxide peaks, gives an accurate determination of the home wavelength.

A scan of 680 nm to 230 nm with a bandwidth of 0.1 nm and step size of 0.1nm can be compared to published NIST data at 25 degrees C.

### 4.5 CD Signal Calibration

CSA, (1S)-(+)-10-Camphorsulfonic Acid, is used to calibrate the CD signal. Two types of tests are conducted: absolute calibration of CD signal intensity and check of the linearity of the CD signal. Both require a fresh solution of CSA.

#### A. Calibration of CSA Solution

##### SPECIFICATION:

Absorbance accurately known to 4 significant figures.

**OBJECTIVE:**

Use an Absorbance measurement to standardize a CSA Solution. By using Beer's Law, it is possible to calculate the product of CSA concentration and cell path length. This product can be used to calculate the ellipticity of the sample.

**MATERIALS:**

1.0 mg / ml (1S) – (+) – 10- Camphor Sulfonic Acid (CSA) in water

Optical grade water

1.0 cm fused silica cell (either cylindrical or rectangular)

Accurate UV-Vis spectrometer.

**PROCEDURE:**

1. Prepare 50 ml of 1.0 mg/ml of CSA solution
2. Use a good quality, calibrated UV-Vis spectrophotometer (such as the Aviv Model 14). Program to scan absorbance from 350 nm to 240 nm every 0.20 nm w/ 1 nm bandwidth, 0.5 averaging time.
3. Run absorbance spectrum of water in cell. Rename as BASELINE
4. Remove water from cell and rinse 3 to 4 times with CSA solution
5. Run absorbance spectrum of CSA solution. Rename as CSA-ABS
6. The absorbance is quite small and baseline drift can significantly change the value, to check for drift examine the BASELINE and CSA-ABS at 320 nm. They should be within 0.001 units of each other.
7. Correct by subtracting BASELINE from CSA-ABS. Create data set CORRECTED.
8. Find the absorbance peak at 285 nm in CORRECTED:

Actual peak: \_\_\_\_\_ nm

If the expected and actual peaks differ by more than 1 nm then either spectrophotometer or CSA is bad.

Abs at peak \_\_\_\_\_

9. Calculate the CD signal of CSA in millidegrees (millidegrees):

$$\text{millidegrees} = 2254.4 \times (\text{Abs})$$

$$\text{millidegrees} = \frac{\text{CD STANDARD}}{\text{CD STANDARD}}$$

The constant 2254.4 is derived from the absorbance extinction coefficient for CSA at 285 nm (335 millidegrees (mg/ml)<sup>-1</sup>) and the CD extinction coefficient for CSA at 290.5 nm: (0.1486 abs (mg/ml)<sup>-1</sup>).

**B. CD Amplitude Calibration****SPECIFICATION:**

CD signal calibrated to within 1%

**MATERIALS:**

Optical grade water

Calibrated 1 mg / ml CSA solution

1 cm Quartz Cuvette

**CONDITIONS:**

Set the CD spectrometer to:

290.5 nm

1 nm bandwidth

20 or 25C if thermoelectric cell is functioning

**PROCEDURE:**

1. First observe the CD signal with no sample. CD baseline should within 1 millidegree of zero. If not then correct air baseline.
2. Fill cuvette with water. CD signal should be within 2 millidegrees of zero. If not then fix cuvette or water.  
CD BASELINE = \_\_\_\_\_ millidegrees
3. Empty water and rinse cell with CSA. Fill cell with CSA and place in sample compartment. CD signal should be in the rough range of 300 millidegrees. If there are large deviations then either the CSA solution or CD instrument is bad.
4. On the OFFSET BOARD adjust "CD CALIBRATE ADJUST" to obtain an observed CD signal to within 1% of the millidegrees value calculated for calibrated CSA solution. Be sure to adjust the target signal for the CD baseline:

CD STANDARD minus CD BASELINE  
= CD TARGET

CD TARGET = \_\_\_\_\_

CD OBSERVED = \_\_\_\_\_

**C. CSA Peak Ratio Test**

SPECIFICATION:

Ratio of 290 to 192.5 nm peaks should be between 1.9 to 2.2

MATERIALS:

0.1 cm path length cell with CSA 1 mg/ml

CONDITIONS:

320 nm to 180 nm wavelength scan  
0.5 nm step  
1.0 nm bandwidth  
Averaging time of 1.0 second

PROCEDURE:

1. Place water in cuvette and measure the water CD baseline. Rename CSABASE.
2. Collect a spectrum of CSA, rename CSA.
3. Subtract CSABASE spectrum from CSA, and name the data set CSA CORRECTED.
3. Find values of peaks values near 290.5 and 192.5 nm.  
192.5 nm CD mdeg \_\_\_\_\_  
290.5 nm CD mdeg \_\_\_\_\_
4. Calculate Ratio  
CD at 192.5 / CD at 290 = \_\_\_\_\_  
Value should be between 1.9 to 2.1.
5. Save files on data disk (CSA, CSABASE).

**4.6 Energy tests**

SPECIFICATION:

Dynode voltages within 25% of typical values.

MATERIALS:

No cuvette in sample compartment.

CONDITIONS:

with high N2 purge (20 SCFH)

1. Record Hours on lamp \_\_\_\_\_
2. Record dynode voltage at 1.0 nm BW

**Table 4.1: Dynode Voltage Profile**

Wavelength	Typical	Measured
800		
700		
600		
500		
400		
300		
250		
225		
200		
190		
185		
180		
175		

**4.7 Noise Measurements**

SPECIFICATION:

Wavelength dependent, see table.

CONDITIONS:

1 nm band width  
600 second kinetic experiment  
4 second time constant  
No cuvette, instrument warmed up 1 hour and purged with nitrogen.

MATERIALS:

CONDITIONS:

Nothing in sample compartment.

PROCEDURE:

1. Go to specified wavelength
2. Record signal as outlined above
3. Save as 500NOISE for 500 nm (etc.)
4. Record RMS noise:

**Table 4.2: RMS Noise Specifications**

Wavelength	Typical	Measured
500 nm	<.0.04	
200 nm	<.0.05	
185 nm	<.0.06	

## Chapter 5: Service and Troubleshooting

### 5.1 Contact Us

In the event that your Aviv CD instrument is not working properly then please feel free to contact us at:

**Phone:** (732) 370-1300 (eastern time zone, USA)

**FAX:** (732) 370-1303

**E-Mail:** [service@avivbiomedical.com](mailto:service@avivbiomedical.com)

Before contacting us please have the following information available:

Name of person to contact

Institution

Telephone number

E-mail address

Instrument serial number

Description of problem

Examples of the problems (data files, test results, configuration files, screen shots, or what ever else helps to characterize the problem)

If possible please use established standards to characterize the problem. Descriptions like “the fitted parameters are wrong” will result in questions about the nature of the sample or the analysis technique.

### 5.2 Problems, Causes and Solutions

Here is a list of common problems, their causes, and their solutions. If further help is needed then contact Aviv Biomedical.

Problem	Cause	Solution
Lamp does not fire	Firing the lamp generates high voltage electronic pulse that can damage the surrounding electronics. A safety circuit is preventing the lamp from firing.	Make sure the CPU / INST PWR ON light is OFF.
	The lamp is so aged that it can not fire.	Replace lamp.
	The lamp power supply is damaged.	Contact Aviv for help.
Lamp turns off	Loss of Nitrogen pressure	Check warning light on lamp power supply. Restore nitrogen supply
	Lamp over heated	Check warning light on lamp power supply. Check fan and cooling fins
	Momentary loss of building power	Shut down instrument, start lamp, and restart instrument
Alarm sounds	Low nitrogen supply	Alarm sounds for 30 seconds or until supply is restored. After 30 seconds lamp is turned off.
CD signal is noisy	Low light level due to excessive light absorbance by sample.	Use shorter path length cuvette, remove salts from buffer
CD Signal is noisy	Low light level due to aged lamp	Replace if >1000 hours

**Table 5.1:** Trouble shooting problems

<b>Problem</b>	<b>Cause</b>	<b>Solution</b>
	Low light level due to aged optics	Remove cuvette and take dynode voltage spectrum. Contact Aviv Biomedical
Temperature never equilibrates	Heat sink has over heated	Use Control Panels: Temperature Control to view the heat sink temperature. If over 30C the check water flow of chiller
	Aged Peltier elements	Contact Aviv Biomedical
Poor CD baseline	Dirty cuvette	Check baseline with water. See Chapter 3:, Sample Preparation for CD Measurements section 3.10, Care and Cleaning of Cuvettes
	Dirty optics	Check for dirt or salt crystals on lens or PMT in sample compartment. Clean with a tissue and water or ethanol
Error message about hitting a limit switch	The wavelength drive has gone too far.	Contact Aviv Biomedical for instructions

**Table 5.1:** Trouble shooting problems

## Chapter 6: Macro Language

### 6.1 Introduction

The reasons for using the macro language are to: 1) automate routine operations; 2) create experiment modes not supported by the routine experiments; 3) have direct control of the hardware. The macro language consists of a set of commands that control the basic instrument functions. By stringing the commands together into a program it is possible to control any aspect of the instrument and have complete flexibility in doing so. This approach makes it necessary to write a command for each function.

The macro language controls the hardware, the setup of experiment parameters, and supports program looping. Functionality seen in high-level program languages is supported. Flow control, decision making, subroutines, math functions, user functions, arrays, and features are not possible.

The macro editor (AMC.EXE, located in C:\Aviv) has each of the commands available in both buttons and menus. Selecting a command opens a panel with the inputs appropriate for that command. This approach avoids having to memorize each of the commands or the command syntax. It is not necessary to know how to program. Rather, experienced users need only to have a list of every step taken to run an experiment, and enter the appropriate macro command in the editor instead to create a macro program.

The macro editor is suitable for all of the Aviv instruments that support the macro language. These are all of the Windows based instruments, including the Models 202, 215, 400 and 410 CD spectrometers, Models ATF-105 and ATFF-212 fluorometers, Model 14 UV-Vis spectrometers, and new models of instruments. Each type of instrument has a different set of commands determined by the hardware,

and the editor must select which command set to use. If the editor is being run on a computer connected to an instrument then the command set selection is made automatically. If the editor is being run on a computer not connected to an instrument then a dialog box will allow the user to select the appropriate instrument type.

This manual is written for the Aviv CD spectrometer, so commands specific to that instrument are described here. The programming concepts are the same though for the other instrument types. Macros files written for one instrument type (CD, for example) can not be used on a different type (like fluorometers), although macros can be used on instrument models of the same type (CD Models 202 and 400, for example). Many macro commands require the presence of accessories (temperature control, for example).

### 6.2 To install Macro Language:

Most Aviv instruments already have the macro editor program installed. An "AMC" shortcut to the editor is usually found in the computer's desktop folder. To install or update the program file:



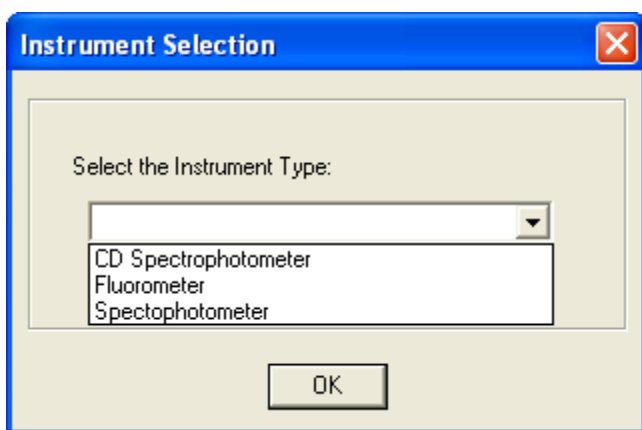
1. Copy all of the specified files into the C:\AVIV\CDS directory.
2. If you have a previous macro language version, you may copy over it. There is no reason to save older, previous versions.
3. You will need to create a SHORTCUT in order for the icon to appear on the desktop.
4. When installation is complete start the program.

## 6.3 Getting Started

### A. Startup

If the editor is being ran on a computer not connected to an Aviv instrument then a prompt will appear asking for instrument information. Select the appropriate instrument type. Older versions of the macro editor had instrument model numbers, but these can be ignored. When the editor first starts there is no macro file program loaded so the bottom portion is blank.

**Figure 6.1:** Instrument Type Selection Panel



### B. Editor Panel

The main portion of the macro editor interface is the editor panel (Figure 6.2: Editor Panel). The first column is the command line number (**Cmd #**). The next column is the **Label**. The label at the top of the list has the version of AMC used to create the macro file, as well the name of the macro file as it will appear in the instrument software. This name is not necessarily the same as the macro's filename (although it can be). The following numbered lines contain the macro commands. Labels are used for controlling the flow of the program when using the loop command, explained below.

The following columns are **Command** and **Comment**. Commands are added by either using the buttons or menus. A command is inserted at the bottom of the list if no insertion point is selected, or below the currently select

command line. When a command is chosen a panel appears with the appropriate inputs and option to enter a comment. When this panel is closed the command appears in the editor.

### C. Buttons

The buttons that appear beneath the menu line, above the command list, are the easiest way to insert commands. The group of buttons on the left side control different physical parts of the instrument (for more information on the hardware please refer to the hardware section of the instrument manual). Buttons in the right show parameters associated with different experiment types. There is a "MISC. CMDS." button in the middle for commands that defy the above definitions. Clicking on a button shows a menu with related commands. Selecting a command opens a panel showing the inputs for the command. Every command has a Comment line can contain any helpful text. The parameters for the command should be filled in before hitting the OK button. Some commands (such as Wavelength or Temperature) have either Absolute or Relative buttons. In this case selecting Absolute will send the device to that absolute value. If Relative is selected then the device will move by the select amount away from the current value (what ever that may be). For example, if a fixed temperature for a titration experiment is desired then the Absolute button should be used. If on the other hand the goal is to increment the temperature in a loop then the Relative button is needed.

## 6.4 File Menu

**Read Macro Files** loads preexisting macro files into the editor. **Save Macro File As** allows users to set the file name and path. Use the filename extension \*.AMC so the file is recognized as a macro program file. Save Macro File saves using the existing filename and path. **New Macro** enables the user to create new macros. **Quit** exits the program.



Figure 6.2: Editor Panel

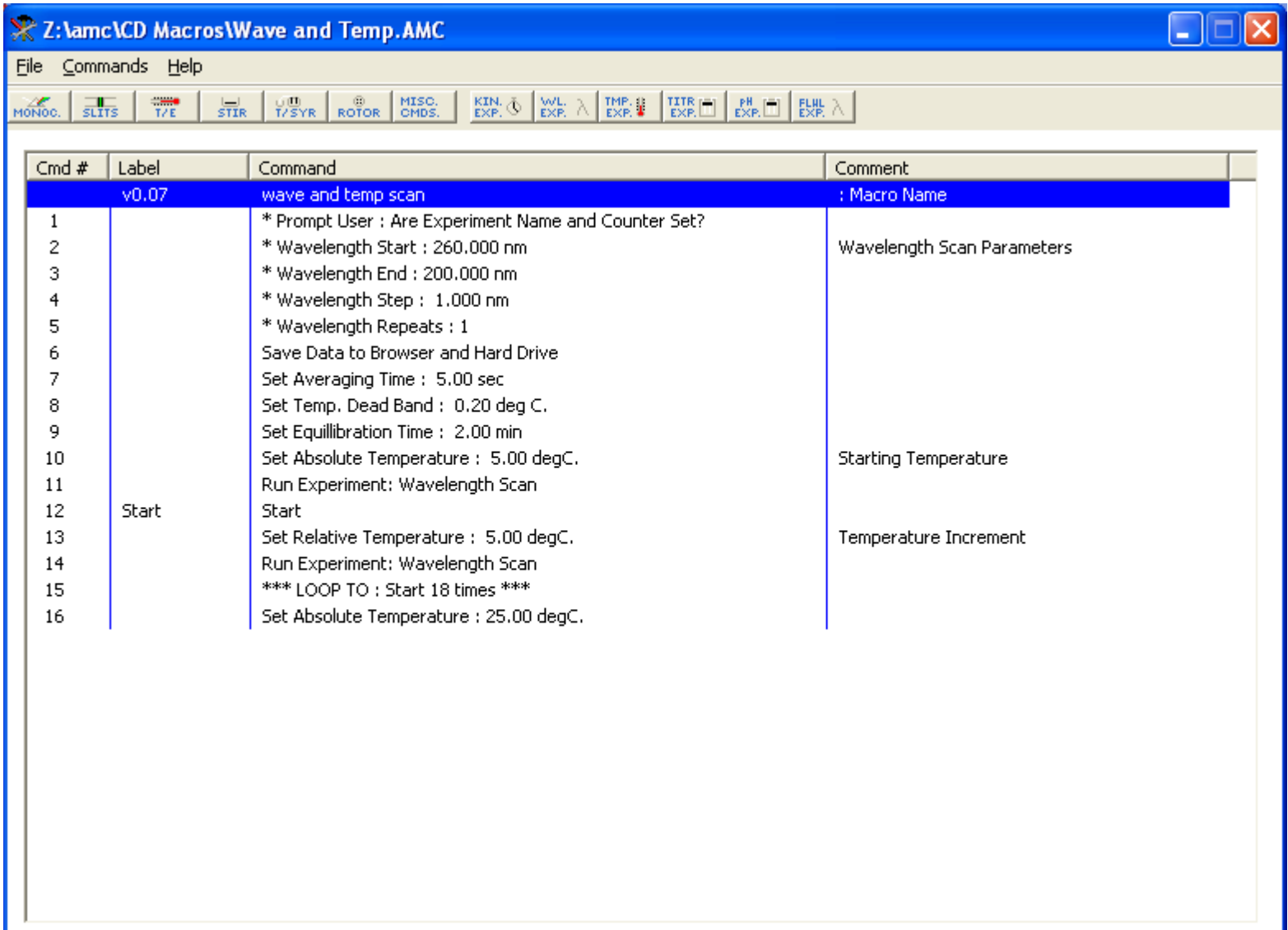
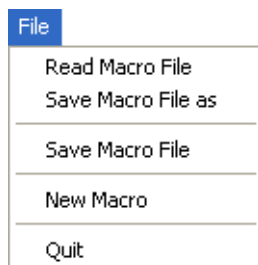
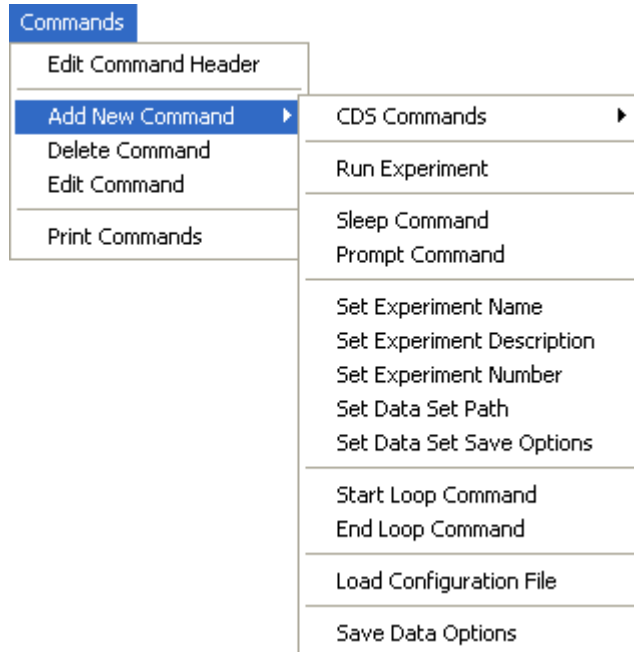


Figure 6.3: File menu



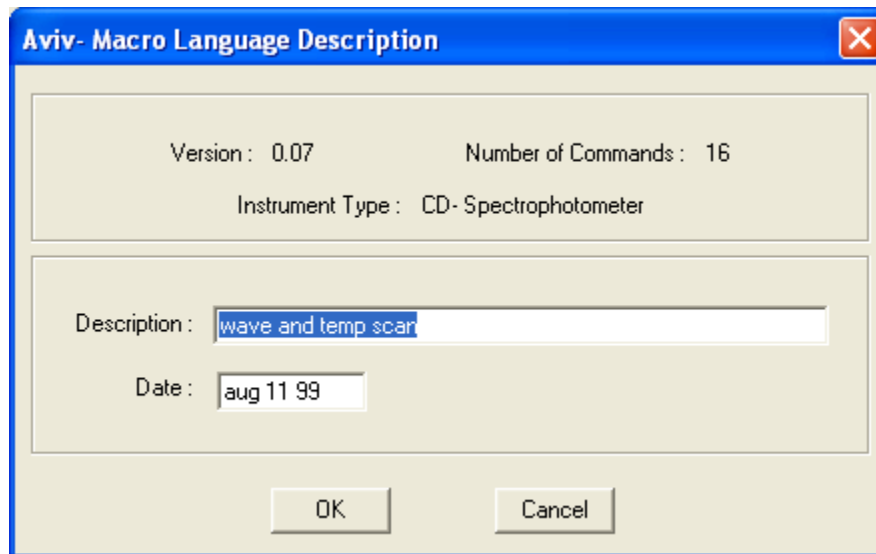
### 6.5 Commands Menu

**Edit Command Header** brings opens the **Aviv Macro Language Description** panel. The **Version** of the editor used to create the macro program file, the **Number of Commands** in the current experiment, and the **Instrument Type** are displayed. The **Description** is used to identify the experiment, particularly in the instrument software. When a macro file is loaded into the instrument software the macro's Description appears at the end of the list of possible experiments that can be ran.

**Figure 6.4:** Commands Menu

The **Date** can be entered as well. You may modify this information at any time by double clicking on the command line.

To use **Delete Command** first highlight the command (line of text) you wish to eliminate from the editor, then click select Delete Command. To **Edit Command** highlight the command line you wish to change and then select Edit Command. A simpler approach is to simply double click on the command you wish to edit. The appropriate command dialog box will appear. **Print Commands** will print the current list of macro commands.

**Figure 6.5:** Macro Language Description

**Add New Commands** expands to show all of the commands available for the instrument. All of the commands available through buttons are available. All of the hardware related commands have the same control as if the user were using the instrument software. These commands are located in the **CDS**

**Commands** submenu. For example, Monochromator Commands: Set Wavelength causes the wavelength to change when executed. Commands of this type should be self-evident. Contact Aviv Biomedical if you

have questions. The following are explanations of commands not found in the instrument control software.

It should be noted that commands directly related to the hardware can be broadly grouped into those that will cause an immediate reaction (the left set of buttons), and those that change experiment parameters (the right set of buttons), and therefore do not come into effect until an actual experiment is ran.

The start of an experiment to collect data is triggered by the **Run Experiment** command. The type of experiment to be ran is selected from the menu list. The experiment parameters used will be the ones currently loaded into the instrument software. If there are no commands to set the experiment parameters then the settings from when the software was started, or later manually set by the user, will be used. If a parameter is set by a command then it is automatically handled when the program is ran. If the parameter needs to be frequently updated (such as the experiment name) then it may be better to not have a command and expect the user to set the parameter before running the macro program.

The **Sleep** command enables the programmer to pause the instrument for a specified amount of time.

The **Prompt** command alerts the user and pauses the program until the user presses "enter".

**Set Data Set Save Options** gives the user a choice of how the data should be saved, including the option of prompting the user when it is time to save the data.

**Save Data Options** gives the options to save data to the data browser or hard drive.

In **Set Data Set Path** you may enter the desired data path to the hard drive.

The loop commands enables allow a sequence of commands to be repeated within the macro program. Clicking on **Start Loop** creates a

label marking the start of a loop. **End Loop** is the ending point of the loop, and must refer to a preceding label in the macro program. When running the macro program will cycle between the start loop label and the end loop label for the number of specified times.

**Load Configuration File** will read in a file containing all of the experiment configuration parameters. A default version of this file is used to save parameters every time the instrument software is shut down, then the same file is read when the software is started again. This way the last setup is remembered for the next use. Within the instrument software the user can create their own versions of configuration files with File: Save Configuration File. that can be loaded. This approach is convenient if a set parameters are always needed. It also saves time and is more accurate than manually or programmatically entering a list of experiment parameters. Another advantage is that the configuration file contains information not available via commands in the macro language. By setting up the parameters in the instrument software, then saving the information to a configuration file, a programmer can set parameters not normally available via the macro editor.

## 6.6 Writing a Macro

The easiest way to write a program is to simply think about the order of steps that are taken when manually running the instrument. For example: after turning on the instrument and inserting the sample; the temperature, wavelength, and bandwidth are set. Next the experiment parameters are set, the experiment name given and perhaps the experiment counter reset. Finally the experiment is started. At completion the data is saved. Perhaps the conditions are then adjusted and the experiment rerun. Each of these functions are possible through the command language. Once a macro program has been written it must be saved to a file. This file is then read

into the instrument control program. The name given in the header appears in the list of available experiments.

## 6.7 Example Experiments

The examples given are for the CD spectrophotometer, however, the concepts are the same for other instrument types. Please pay attention to the concepts and not necessarily the command details.

### A. Example 1: writing a new program

Start the macro editor ("AMC" on the screen desktop). Go to the **Commands** menu, **Edit Command Header** and enter the "Demo1" for the **Description**. This is the name that will appear in the list of possible experiments in the instrument software. Next enter the commands needed to initialize the experiment parameters. This macro program will run wavelength experiments, so to set the bandwidth select the menu options **Commands: CDS Commands: Slit Commands: Set Bandwidth** or simply hit the **Slits** button and select **Set Bandwidth**. Enter "1" for the bandwidth and enter the command. Press the **WL. EXP.** button to see the list of wavelength experiment parameter commands. The goal is to scan 320 nm to 200 nm with a 1 nm step size. Select the **Starting WL** command and enter 320. Next select **Ending WL** and enter 200. Select **WL Step** and enter 1 for the wavelength step size. We want to make only one scan; so we're going to select the **Number of Scans** command and enter "1". Next, select how to save the data. Click on the **MISC. CMDS.** button and select **Set Data Set Save Options**. The Data Set Save Options Box appears (with the same options as the instrument software). Select options to save the CD Signal and CD Dynode Voltage. Also under the **Misc. CMDS.** button select **Save Data Options** and select how to save the data to disk.

The experiment is now configured but at this point no command has been given to actually collect the data. Click the **MISC. CMDS.** button

and select **Run Experiment**. Select **Wavelength Scan** from the list. Finally, save the experiment as DEMO1.AMC with the **File: Save. Macro File As** menu option.

Start the instrument software and use the **File: Read Aviv Macro File** menu to select and load the new macro program. Click on the **Configure Experiment** menu and click on the **Type** list. "Demo1" should appear at the end of the list. Select it, then click the menu item **Exit/ Save Configuration**. In the **Data Collection Panel** the **Experiment Type** is now **Macro Exp**. The contents of the macro file can be viewed (but not changed) with the menu **Displays: Macro Command Window**. Clicking on the **Run Experiment** button will start the macro experiment. The Command Window will open to show the experiment progress.

### B. Example 3: wavelength and titration

A library of pre-existing macro programs is shipped with Aviv instruments. Figure 6.6: Titration Experiment shows an example of a "multi-dimensional" experiment that collects wavelength spectra over a range of concentrations. A syringe pump is used to make small injections, such as for a ligand titration experiment. The strategy used here is to rely on the user to first set up some of the experiment parameters that are frequently changed. Command one displays a dialog to remind the user of what is expected. Command two again displays a dialog telling the user that the syringe pump should have already been loaded with the appropriate solutions. Commands three and four determine what data is to be saved and when it should be saved to the hard drive. Commands five to eight initialize the wavelength experiment parameters. Parameters the user may have entered before the macro program is ran might be changed by these commands.

Command nine sets the thermal equilibration time before data is collected. At the beginning of all wavelength experiments a temperature

Figure 6.6: Titration Experiment

Cmd #	Label	Command	Comment
	v1.02	Wavelength and Titration	: Macro Name
1		* Prompt User : Are the Name, Counter, and Data Path Set?	The user should have already enter info
2		* Prompt User : Pump should be Primed, and Temperature Set.	Pump should already be ready.
3		Set Data Set Save Options	
4		Save Data to Browser and Hard Drive	How to take dispose of the data
5		* Wavelength Start : 320.000 nm	Initialize WL experiment
6		* Wavelength End : 200.000 nm	
7		* Wavelength Step : 1.000 nm	
8		* Wavelength Repeats : 1	
9		Set Equillibration Time : 0.50 min	Used as the Stirring Time
10		Titrator #1 LEFT Valve: Set to INPUT	Initialize syringe pump
11		Titrator #1 LEFT Syringe: Absolute Amount 500.00 ul.	
12		Titrator #1 LEFT Valve: Set to OUTPUT	
13		Stir Speed : 100.0	Set stir speed
14		Run Experiment: Wavelength Scan	Initial Spectra
15	start1	start1	First titration loop
16		Stirrer : ON	
17		Titrator #1 LEFT Syringe: Relative DISPENSE 5.00 ul.	Make small injection
18		Run Experiment: Wavelength Scan	Collect WL spectrum
19		*** LOOP TO : start1 10 times ***	Make 10 small injections
20	start2	start2	Second titration loop
21		Stirrer : ON	
22		Titrator #1 LEFT Syringe: Relative DISPENSE 10.00 ul.	Make larger inejction
23		Run Experiment: Wavelength Scan	
24		*** LOOP TO : start2 5 times ***	
25		* Prompt User : Finished!	Get user's attention

equilibration period is enforced, and this command sets the duration. In this particular situation though the temperature is not being changed and the equilibration time is probably not needed for this purpose. However, a solution mixing period is needed after every injection. Although not explicitly stated, the solution will be mixed via the temperature equilibration period at the start of every wavelength experiment.

Commands ten to twelve move the syringe valves and plungers to appropriate positions. When writing macro experiments there is no guarantee that the syringe pump will be in any given state, so explicit commands to initialize the valve and plunger positions are necessary. Command ten sets the valve to the input position so that additional solution can be drawn in or out from the reservoir (not the sample). Command eleven makes sure that 500 ul are present in the syringe. Command twelve sets the valve back to the tubing connected to the

sample, ready for injections. Command thirteen ensures the stir speed is ready, then command fourteen collects the first spectrum before injections are made. Now the titration can start.

Command fifteen's "start1" label marks the start of the first loop. The stirring is forced on with command sixteen, an injection is made, and a wavelength experiment is started (beginning with the 30 second equilibration time). When data collection is finished the loop counter is incremented and the loop repeats for nine more times.

After 10 small injections the optical signal is not changing so fast, so larger sized injections are desirable. The "start2" label marks the beginning of a series of 10 ul injections and data collection. When this series is finished a final prompt is given to call attention that the experiment is over. Sixteen spectra will have been collected: the first one before any injections, ten spectra after 5 ul injections, and five more spectra after 10 ul injections. A total of 100 ul will have been injected.

## **6.8 Macro Program Library**

A library of macro files has been collected and is available for all Aviv users. The files should be located in the C:\Aviv directory, possibly in a subdirectory. They will have the file extension \*.AMC. If they are missing, an explanation is needed, help editing the files is needed, or for what ever reason help is desire, then contact Aviv Biomedical for support.

## Chapter 7: Thermoelectric Cell Holders

### 7.1 Introduction

The Aviv thermoelectric cell holders are sold as accessories, but are common to many Aviv instruments. Several common are discussed in detail here: **Single Position**, **Total Fluorescence** and **Five-Position**. Other designs exist and follow the same ideas, although the form may vary.

The designs of all thermoelectric cell holders are based upon the same basic principles. The sample cuvette sits in a thermostated Jacket. Built into the Jacket is a temperature probe that is used to measure the sample temperature. Attached to the outside of the Jacket are Peltier Elements. These behave like heat pumps, driving heat into, or out of, the Jacket (and sample). The direction of the heat flow depends on the direction of the applied electrical current. Beneath the Jacket is a Stirring Motor, whose speed is controlled through the instrument software. Built into the Jacket are ports through which Nitrogen gas can be flowed. The ports are positioned to blow the nitrogen onto the cuvette windows. This feature is useful when the cuvette is at low temperature and might otherwise form condensation in the optical path. On the outside of the Peltier Elements are Heat Sinks. Water flows through the Heat Sinks to remove waste heat. The chiller water temperature should be set to ~20C constant. All thermoelectric cell holders work with 10 by 10 mm rectangular path length cuvettes, as well as shorter path length cuvettes when used with the appropriate cell spacers.

The **Single Position** Cell Holder is the simplest design. It is useful for wavelength, temperature and kinetic experiments. With the appropriate accessories it can also be used with titration and pH titration experiments. The **Total Fluorescence** Cell Holder has all the same capabilities as the Single Position, but

also has the ability to measure fluorescence. The **Five Position** Cell Holder is capable of holding up to five cuvettes during experiments. It is suitable for wavelength, temperature and kinetic experiments.

It should be noted that the different Aviv spectrometers use the same types of thermoelectric cell holders, although the sample compartment dimensions may be different. The pictures show are for the Aviv CD Spectrometers, although the same basics apply to the Aviv UV-Vis Spectrometer as well.

### 7.2 Installation

Instructions for exchanging cell holders

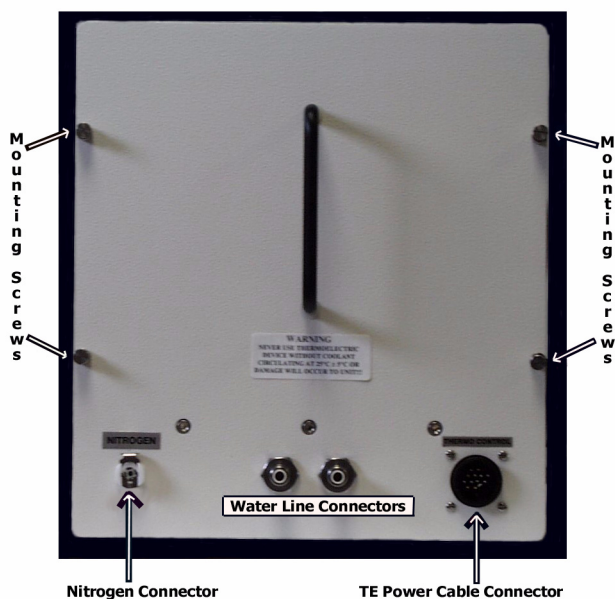
1. Turn OFF power to instrument.
2. Remove the existing cell holder from the sample compartment if present. Remove cables and hoses if present. Unscrew the four mounting screws on the front of the cell holder. In most cases pull the cell holder straight out through the front of the instrument. Be sure to support the cell holder's weight when the back edge falls out of the instrument.
3. Put the back edge of the cell holder on the top of the front pair of rollers in the sample compartment. If the cell holder is a **Five-Position** type then a motor hangs too low to allow this step. Instead, remove the cover from the top of the cell holder, pass the unit into the sample compartment so the motor clears the bottom edge, then lower the cell holder so the notches on the side edges clear the rollers.
4. Gently roll the accessory into the sample compartment.
5. Screw down the 4 mounting screws in the front panel to fasten the accessory to the instrument.

6. Align and screw down the thermoelectric power cable into the connector. Be sure to use the cable that was supplied with the accessory. Different types of cell holders require different cables, so pay attention if there is more than one type of cell holder present.

7. Push the water lines firmly onto their connectors. These are locked into place when a click is heard.

8. If working at temperatures below ambient, there is a risk of water vapors condensing on the cells in the optical path. Dry nitrogen can be supplied through the Nitrogen Connector to keep the cells dry. The line is located under the instrument and is controlled at the instrument's nitrogen manifold. This can also be used to purge the sample compartment when working in the deep U.V. Plug the hose into the

**Figure 7.1: TE Door**



Nitrogen Connector.

9. If the **Total Fluorescence** Cell Holder is being installed then the PMT Cable should be attached inside the sample compartment.

10. If the **Five Position** Cell Holder is being installed then the connector on the front panel for the motor drive must also be connected.

### 7.3 Total Fluorescence Cell Holder

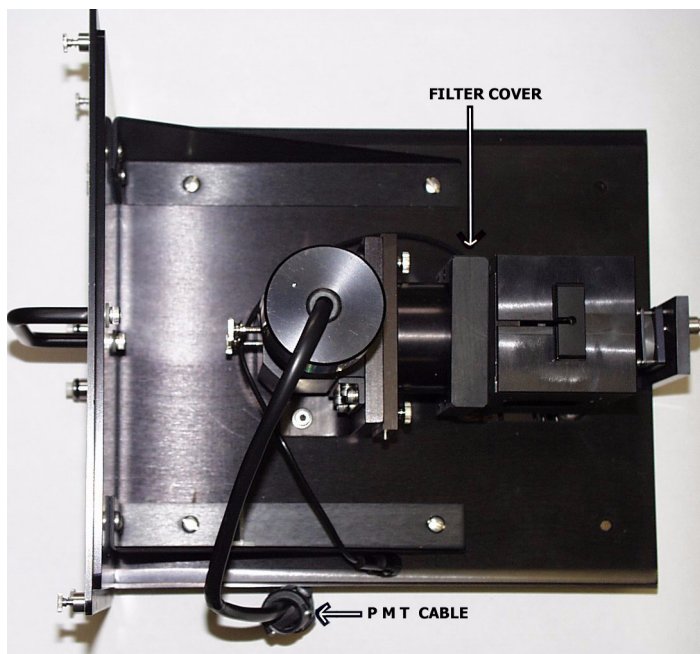
The Total Fluorescence cell holder is a modification to the 1 cm single position cell holder. This accessory is available for the Aviv CD spectrometer only. A photomultiplier tube

(PMT) sits at right angles to the excitation light beam to measure scattered and fluorescent light.

#### A. Glass Filters

A glass light filter holder sits between the sample and PMT. Aviv supplies the total fluorescence TE with 305 and 320 nm cut-off filters, although others can be used. The wavelengths were chosen to work with proteins, which are typically excited with 280 nm light. These allow light of longer wavelengths to pass through, while absorbing shorter wavelengths. They serve the function of removing scattered light. Selection of the appropriate filter is important to obtain the right results. The



**Figure 7.2:** Top View of TE

ideal filter should pass fluorescent light while absorbing scattered light. There are a number of filters available, however selection of the appropriate filter is very important. For example, if the sample undergoes a change in its quantum yield then it is appropriate to monitor the total fluorescence. A typical example would be if the sample is excited at 280 nm and fluoresces at 330 nm with different intensities depending on the physical state. In this case the 305 nm cutoff filter is appropriate. This filter will allow nearly all of the fluorescent light to pass while absorbing nearly all of the scattered light. On the other hand, if the sample's fluorescent intensity does not change, but instead shifts from a maximum of 330 to 340 nm then the 305 nm filter will see no change in the signal. The 320 nm cutoff filter would be a better choice because it will remove some of the 330 nm light while letting the 340 nm light pass. There are numerous filters available commercially to meet your specific needs.

#### **B. Fluorescence PMT**

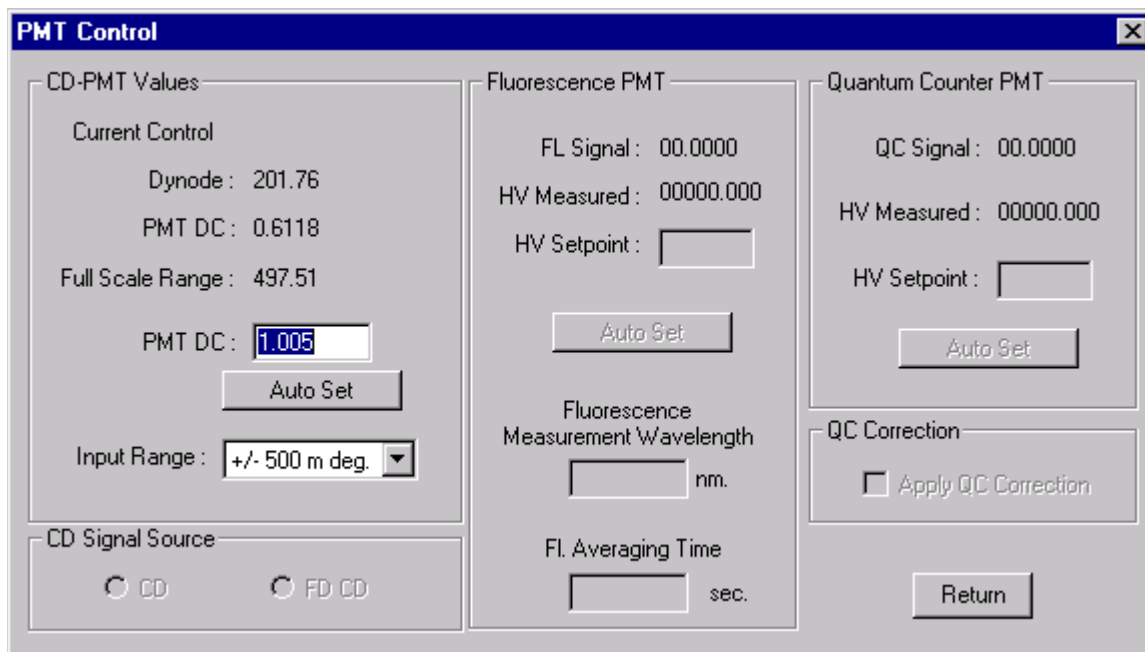
Control of the fluorescence PMT is done through the **Control Panels** Menu **PMT Control** Window. Full scale for the fluorescence signal is 0 to 5. The fluorescence is a separate detector from the CD PMT. By changing the **HV Setpoint** the PMT sensitivity is adjusted. The monochromator should first be set at the wavelength that will be used for fluorescence measurements. Make sure auto slit feature is turned off. The optimal voltage will depend on how fluorescent the sample is. The user can manually set the HV Setpoint at about 200 volts, then raise it until the signal shown in the "**FL Signal**" Field is in the range of 0.1 to 1.0 intensity. Full scale for the high voltage is 0-1200, although the optimal range is 180-1000. Remember that fluorescence is a relative measurement. The **Actual PMT Voltage** is the voltage currently applied to the PMT.

### C. Automatically Setting Fluorescence PMT High Voltage

Fluorescence measurements can be made for all experiment types. The fluorescence PMT high voltage can also be automatically set. First enter a value for "**Fluorescence Measurement Excitation Wavelength**". Next, click on the "**Auto Set at Excitation WL**"

button. The high voltage will automatically be ramped up or down to give a fluorescence signal close to 1. If the correct voltage is far from the starting point, then it will be necessary to use Auto Set more than once. Wavelength scans will collect the excitation spectra. There is no emission monochromator (filters are used instead) so emission spectra are not possible.

**Figure 7.3: PMT Control Panel**



### D. Simultaneous Measurements

The fluorescence and CD PMTs do not share signal electronics. This enables the user to perform CD and fluorescence measurements simultaneously. Stopped-flow experiments can measure fluorescence and CD at exactly the same time and wavelength. The user may also perform Titration and Temperature Melts by monitoring the CD at one wavelength, while measuring the fluorescence at another. The exceptions to this rule are wavelength and stopped-flow experiments where either the wavelength is being scanned or the chemistry is too fast to change the wavelength.

## Chapter 8: Titration Accessory

### 8.1 Introduction

The Aviv Automated Titration System is designed to automatically perform ligand and solute denaturation experiments. It is also required for the automated pH titration accessory. These types of experiments can be done manually, however the process can be laborious. For each measurement the user must at least open the sample compartment, remove the sample, make an injection, stir, replace the sample and make the measurement. Besides being time consuming this process can introduce errors in the measurements due to varying cuvette positions in the sample holder, user error in the injection volume, possible fingerprints on the outside of the cuvette, dust that falls into the cuvette, et cetera. The Aviv automated titration system can add a precise volume to the cuvette, mix and measure automatically. Ligand titration and solute-induced denaturation experiments are discussed separately because of a number of differences in the methods and the intentions behind the two types of experiments. pH titrations are discussed in a separate section devoted to it. This part of the manual is principally devoted to the hardware. Detailed explanations of the software are explained in greater detail in Chapter 2: Software Details.

#### A. Ligand Titrations

Ligand titrations are intended to add to the sample cuvette some substance while observing signal changes brought on by a chemical change. The titrant added frequently is a small molecule (ligand) that binds to a larger molecule (macromolecule) already in the cuvette, however this arrangement can vary. Normally an optical signal sensitive to the physical states of the macromolecule is measured, and changes in this signal indicate when the ligand binds to the macromolecule. To obtain thermodynamic data, such as binding

constants, the concentrations are kept in the range of the binding constant so that both free and bound species are present during the titration. This requirement means that the ligand concentration in the cuvette is on the same order as the macromolecule, and the volume of ligand added is small in comparison to the total sample volume. The added volume will dilute the macromolecule, but corrections for concentrations can be applied in the subsequent data analysis.

### 8.2 Solute-Induced Denaturation

In the case of solute induced denaturation experiments the solution being added does not contain a ligand, but rather some denaturing solute that destabilizes the macromolecule's structure. Common denaturants are urea or guanidine hydrochloride (GuHCl). The concentration of denaturant required in the sample can be quite high, on the order of several molar. The maximum solubility of the denaturants is 10 M for urea and 8 M for GuHCl, so to achieve the appropriate concentration in the cuvette a large volume of denaturant must be added. This additional volume will seriously dilute the macromolecule and possibly overflow the cuvette. To correct for the first problem a common technique is that of "the method of Job". To avoid diluting the sample the same concentration of macromolecule in the denaturant is matched to the macromolecule concentration in the cuvette. **This approach requires that the denaturation process be reversible**, because macromolecules in the concentrated denaturant must be capable of refolding when injected into the sample solution. The problem of overflowing the cuvette is handled by the Aviv automated titration system. Normally the left syringe of the Hamilton pump is filled with ligand or concentrated denaturant. For ligand titrations only the left syringe is used to add titrant. However, for

solute induced denaturation experiments the right syringe is filled with buffer and used to remove sample. The selection for these options are the “Increasing” and “Constant” buttons under “Syringe Volume” in the Titration Experiment configuration panel. The software automatically calculates the appropriate injection volume, and before every injection removes that volume from the cuvette using the right syringe. The concentrated denaturant is then added to bring the sample back to its original volume. The entire titration is performed in this manner, so the volume is held constant while the denaturant concentration is raised. See Section 2.4 Experiment Configuration Panels for more information about setting up titration experiments.

### 8.3 Helpful Hints for Titration Experiments

Any time an injection is made into the cuvette there must be some stirring action to keep the sample homogenous. The Aviv thermoelectric cell holder will provide the necessary stirring, as well as tightly regulate the temperature. The stirring speed must be set to give adequate mixing, but not to create artifacts (see 2.7 Control Panels, subsection F. Stir Control). The small “flea” stir bars are OK for ligand titrations, but solute denaturation experiments require more strenuous mixing. In these cases it can be difficult to push the dense, viscous denaturant up from the bottom of the cell. If the mixing is not adequate then the denaturant will collect at the bottom of the cell and an upper layer of nearly undisturbed sample will develop. In this case the experiment will be worthless because the concentration of denaturant in the light beam is unknown. For solute denaturation experiments we recommend that the bulky, cross shaped stir bars be used (Figure 8.1: Recommended Stir Bar). These generate a stronger mixing action and the large size helps by displacing sample. Care must be taken so that bubbles are not in the light path during optical measurements. This



**Figure 8.1:** Recommended Stir Bar

can be avoided by turning OFF the option to “Stir During Measurement” in the Stir Control panel. A “Delay Time” must be set for several seconds so that after the stirring is stopped there is sufficient time for the bubbles to leave before measurements are made. With this approach the stirring can be set to the maximum speed without risk of interfering with measurements. The sample volume can influence the mixing. Obviously the meniscus must sit above the light beam but too large a sample volume is more difficult to mix. In our experience 1.8 ml of sample is best for the cross stir bar in the one by one centimeter cells.

The syringe pumps used by the Aviv automated titration system can deliver a wide range of volumes, but there are limits. The motion of the plungers is controlled by stepper motors, which move in discrete steps. For any given syringe size there are 2000 steps available, so the smallest increment that can be delivered is 1/2000 of the total syringe size. For example, with a 500 ul syringe the resolution is 0.25 ul. Smaller volumes are rounded up or down. To ensure that the round-off error does not adversely affect the injections we suggest that the smallest injection size be kept to greater than 1/20 of the syringe size (at least one hundred steps per injection). If smaller injection volumes are required then a smaller syringe size is recommended by the software. These can be purchased from Aviv Biomedical. The software is designed to make the entire injection with a single stroke. If there is not enough solution in the syringe then the syringe will automatically be refilled. If the

injection volume is larger than the syringe size then the injection will be made in two or more passes.

Diffusion of material from the tip of tubing is another concern. The injection tubing has been drawn out to make the opening smaller, but liquid in the end of the tube is still exposed to the sample. In the case of dense, viscous solutions (such as 8 M guanidine hydrochloride or 10 M urea) the tubing tip has been turned upward to prevent titrant from dribbling out. In this case the injection volumes are large and diffusion is insignificant. In the case of ligand titrations the injection volume may be very small (on the order of a microliter). In this case diffusion could prematurely release solutes into the sample. This problem can be reduced by making the titrant less concentrated, and making larger injections. Injections of 10  $\mu$ l or larger work well.

The tubing that fits in the cuvette obviously must be cleaned between experiments. Frequently the tubing is wiped with a tissue to dry it. Many tissues are not lint free (including "lint free" KimWipes) and this can lead to noise in the measurements. Any lint present on the tubing will come off in the sample and pass through the light beam. The lint will scatter light and probably will deteriorate the measurements in other ways too. The best way to dry tubing is with lens paper (even though it absorbs liquids poorly) or to blow it dry with a stream of nitrogen gas.

#### 8.4 Installing Pump Hardware

Installing the titration accessory is fairly easy. The syringe pump should be placed on top of the instrument, next to the sample compartment.

- 1) Follow the Hamilton syringe pump manual for connecting the valve, syringes, and power cord. Consult chapter 2, pages 9-15.
- 2) The pump and computer should be turned off. It is good to confirm that the dials and switch on the back of the pump have not been

moved during shipment. The correct positions are SWITCH in the UP position, BAUD is 3, and UNIT # is 0. The serial communication cable should be plugged into the "RS-232 IN" port of the pump and an available serial port of the computer (usually found on the serial extension box, plug # 5).

- 3) The software should be configured to communicate with the syringe pump. Click on Control Panel, Instrument Configuration, I / O Setup. Under COM Ports select the appropriate comport (usually COM 7). In a typical configuration COM port 1 and possibly 2 are found on the back of the computer. COM 3 and above are the ports on the serial expansion box.

- 4) The Hamilton tubing (serial number 240010 or 240000) should be attached to the left and right side ports of the valve head. The tubing for the center ports of the valve head have been modified to fit into the Aviv instrument. They already are attached a the titrator cuvette cap. The tubing with the curled tip should be attached to the left-center port, and the tubing with the straight tip goes in the right-center.

The tubing can behave like fiber optics and route stray light directly into the cuvette. To prevent this problem a light trap has been provided. The following instructions are for mounting the light trap to the instrument:

- 5) On the top edge of the CD sample compartment is a notch, filled with a plastic plug. Remove the plug.
- 6) The light trap is supplied with all necessary the parts connected in the correct positions. Remove the two round headed screws on the light trap (note that the longer screw has the wire loop attached to it) and detach the notched plastic plug.
- 7) Slide the notched plastic plug into the notch in the sample compartment top, and insert the two round headed screws, with the wire loop positioned over the top of the notch.

8) Reassemble the light trap inside the sample compartment, beneath the notched plastic plug. Tighten the two round headed screws.

9) The tubing can be positioned now or left till later. The side of the light trap has a cover held in place with two thumb screws. To place the tubing in the light trap first remove the back thumb screw that holds the cover on the trap. Loosen, but do not remove the front thumb screw. Starting at about the middle of the tubing, place the tubing into the notches of the plastic plug and light trap. Once the tubing is around the 180 degree turn let the lid hang down. The edge of the lid will hold the tubing in place while the tubing is positioned around the back turns. Swing the lid up, make a last check that the tubing is in the right places, and tighten down the thumb screws. The wire loop is included to hold the tubing in place, and to provide strain relief. The tubing can be slipped into the center of the loop by pushing it into the groove between the loops, starting at the bottom, and working to the top. It may be necessary to remove the tubing for cleaning. Simply reverse the process to remove the tubing.

## 8.5 Setting up a Titration Experiment

### A. Loading the Pump and Cuvette

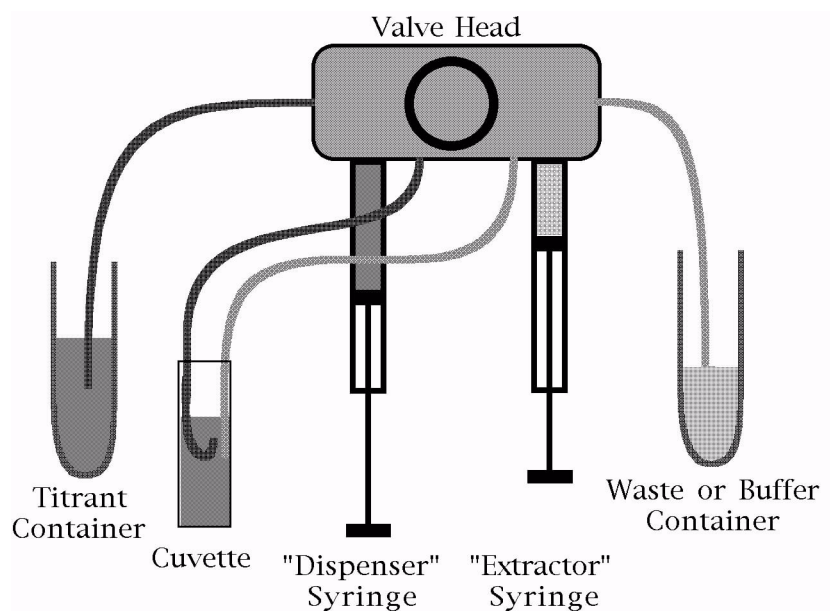
Gas solubilities decrease with increasing temperatures. If a solution is taken out of the refrigerator and immediately loaded into the syringe pump then bubbles will almost certainly appear later. The bubbles come from atmospheric gases that had dissolved in the cold solution. The best approach is to degas solutions while they are allowed to warm up. Solutions can be degassed by placing them in a bell jar connected to a water aspirator for 15 minutes. Titrant (ligand, denaturant, acid or base) is loaded into the left syringe, and is injected into the cuvette through the curled tubing. If two syringes are used then the right syringe is loaded with buffer or water. The contents of the right syringe are not injected

into the sample, but are needed so that the plunger has something hard to work with. Air is compressible and its volume can not be accurately controlled. It is suggested that the right syringe be filled first, so that if a mistake is made during loading then valuable titrant will be less likely to be involved. The syringes are filled and purged using the **Step/Prime** button on the front of the Hamilton syringe pump. The Step/Prime button will purge the pump by moving solutions in from the two side ports, through the syringes, out the two center ports and through the cell cap. The cell cap can be positioned above the titrant and waste containers to recycle solutions. It is necessary to cycle the syringes several times to remove all air bubbles. During experiments the flow of titrant continues in through the left side port to the left syringe and out the left center port to the syringe. If the right syringe is used then its flow is reversed and sample moves from the cuvette, through the right syringe and out the right side port to the waste container.

Once the pump has been filled place the tubing into the light trap (if not already there) and the cell cap into the sample compartment. Put the stir bar in the cuvette and add sample. Put the cuvette cap on the cell. The hook is best positioned on the back wall of the cuvette. Make sure that the tubing is located in the corners of the cell. The goal is to position the tubing well into the sample but so that the light cleanly passes through the middle.

### B. Cleanup

At the end of the experiment the cell should be removed and examined for complete mixing of the sample. The cell and stir bar can be cleaned as usual. The syringe pump can be cleaned by first pumping out the solutions, then rinsing with water, then pumping through a laboratory detergent (not SDS). The detergent should be rinsed out with large amounts of water. The syringes can run continuously by setting the **Prime** switch. When the lines are clean the syringes should be removed from the



**Figure 8.2:** Syringe Pump

pump, and washed out separately. The system can then let to dry in the air. If there is a small amount of water left in the lines it can be blown out with nitrogen gas, or flushed out with sample and buffer at the beginning of the next experiment.

## 8.6 Conclusions

Obviously this system is not trivial to learn. There are a number of details, and they are best learned by practice. Therefore we suggest that the users first perform experiments with water and a marker (CSA is good for CD measurements, L-tryptophan is good for fluorescence) only. Later, practice with inexpensive reagents with known transitions. After the user is familiar with the whole operation then the real experiments can be started.

## Chapter 9: Stopped-Flow

### 9.1 Introduction

The stopped-flow accessory is designed for the rapid mixing of reagents to measure kinetics on the millisecond and longer time scales. The principle components of the stopped-flow accessory are the Syringe Drive Tower, the thermostated Cell Holder, the Mixer / Optical Cell assembly, the Stopped-Flow Power Supply, and the Thermoelectric Power Supply. The Syringe Drive Tower is designed to deliver aliquots from multiple, independent syringes to the mixer in milliseconds. From the mixer a homogeneous solution flows to the optical cell, where optical measurements are made. These components are integrated through the software so the whole package behaves as a single instrument.

### 9.2 Parts List

The stopped-flow accessory includes the following parts:

Syringe Drive Tower (1), including:

- 1 ml Syringe (1)
- 2mlSyringe(1normally,2ifdrive tower supports 4 syringes)
- 5 ml Syringe (1)
- ReservoirCaps(3normally,4ifdrive tower supports 4 syringes)
- Light Trap (1)

Thermoelectric Power Supply (1) (used by all temperature controlled cell holders), including:

- TemperatureControllerPower Cable (1)
- Serial Communication Cable (1)
- Power Cord (1)

Stopped-Flow Power Supply (1), including:

- Controller Card (1)
- Motor Power Cable (1)
- Motor Signal Cable (1)
- Ribbon Cable (1)
- Power Cord (1)

Cell Holder (1), including:

- Cell Adaptor for 1 cm cells (1)
- Alignment Target (1)
- Assembly Cover (1)

Mixer / Optical Cell Assembly (1), including:

- "T" Mixer (1)
- "Delta" Mixer (1)
- "Fersht" Mixer (1)
- Tubing (4)

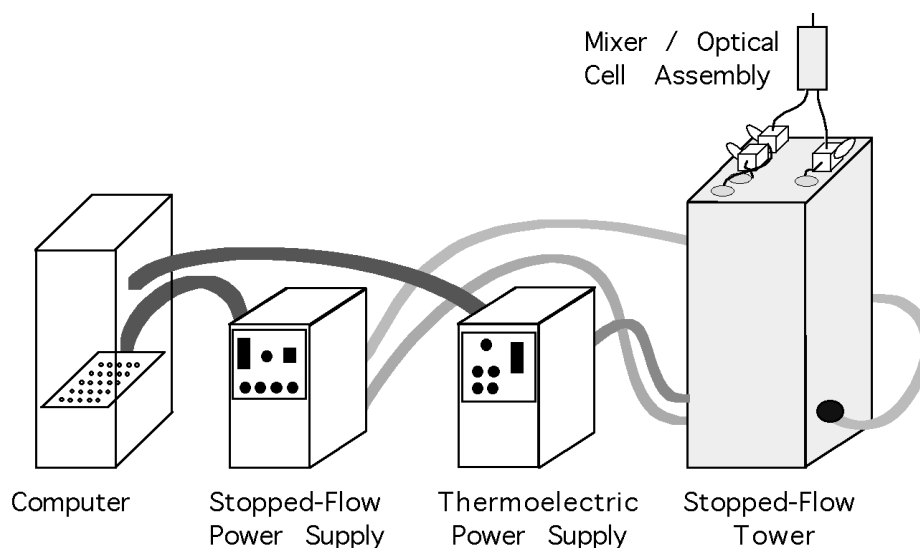
"T" Connector (1)

- 25 ml Waste Syringe (1)
- Miniature Torque Wrench (1)
- Miniature Wrench (1)
- Plastic Disposable Pipettes

### 9.3 Overview

The principle components of the Stopped-Flow accessory are the Motor Controller card in the Computer that is connected to the Stopped-Flow Power Supply through the Ribbon Cable. The power supply has two cables that transmit power to the motors and read back the positions. The Thermoelectric Power Supply communicates with the computer through an RS-232 line. A cable sends power to the Peltier elements in the Cell Holder Assembly and the Syringe Drive Tower. Temperatures measurements in these locations are sent back to the controller which then adjusts power to achieve the user specified temperatures. Inside the Syringe Drive Tower each syringe is connected to a separate motor allowing independent control of the fluid flow rates. At the top of the tower are valves connected to each syringe as well as reservoirs and the mixer. The syringes are filled and purged with the valves set to the reservoirs. When an experiment is run the valves are switched to the mixer. The fluid stream from the mixer passes through the optical cell where measurements are made and out into a waste syringe.





**Figure 9.1: Stopped-Flow Layout**

Data collection typically begins when the motors begin moving. The optical signals are processed by the instrument's electronics and sent to the computer where an Analog to Digital (A/D) board converts the voltage levels to numbers. This is done every 10 microseconds, so the signals are greatly oversampled. These numbers are then averaged by the software to produce the data points requested by the user.

Routine use of the stopped-flow is through the same user interface software that controls the rest of the instrument. The software is explained in a separate chapter. Utility software for calibration and diagnostics is explained in the Cleaning and Maintenance section, below.

## 9.4 Hardware Components

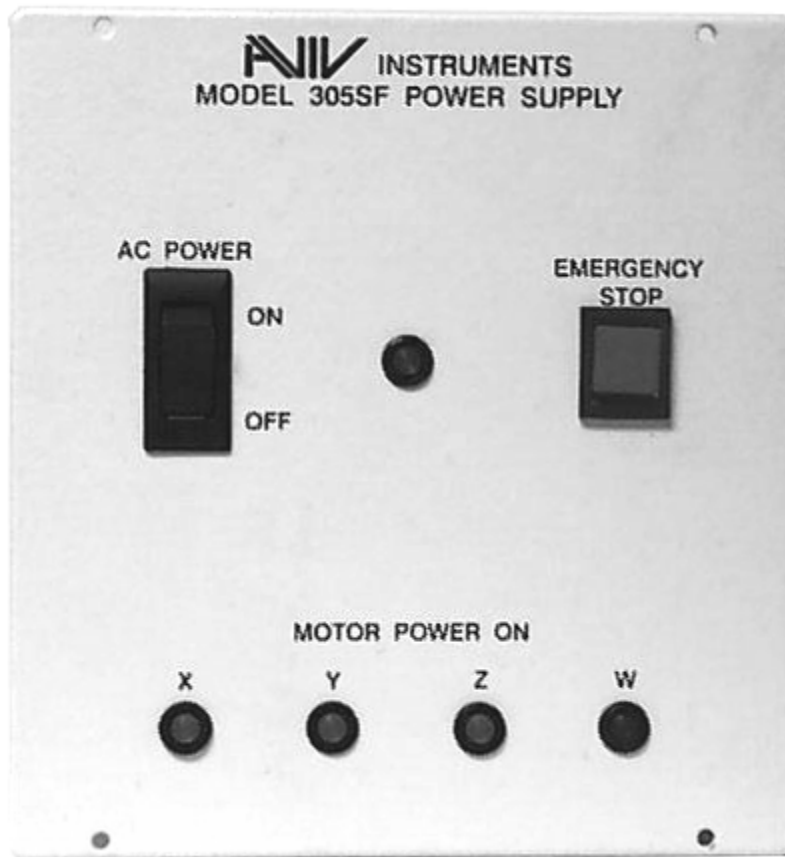
### A. Stopped-Flow Power Supply

The stopped-flow power supply receives signals from the controller card and relays that information to the motors. Encoders attached to the motors report back the positions to the power supply, which passes that information to the controller card. On the front panel the **AC Power** turns control power to the box. Next to

that is a power indicator light that comes on when there is AC power. This is fed to a DC power supply that is separately switched on and off under control of the software. The DC power can also be shut off with the **Emergency Stop** button on the front panel and a safety circuitry built into the power supply. If the power supply detects that too much power is being delivered to the motors then the DC power is automatically turned off. When DC power is being delivered to the motors then the **Motor Power On** lights will come on.

### B. Stopped-Flow Drive Tower

Several items attach to the outside of the tower. It is attached to the instrument through a hydraulic jack, used to raise and lower the drive in and out of the sample compartment. The handle for the jack is located beneath the power switches. The handle can be pulled to swing it in and out of position. The tower has three plugs on its sides: one for motor power, one for position feedback and one plug for temperature control. The top of the tower contains the reservoirs for loading reagents and valves to direct the flow of reagents. Syringes sit beneath the valves.



**Figure 9.2:** Stopped-Flow Power Supply Front Panel

Inside of the syringe tower are DC servo motors, each connected to a single syringe. Power for the motors is supplied by the Stopped-Flow Power Supply. Position control is achieved through optical encoders on the motor shaft. Signals from the encoders are sent to the motor controller card, which adjusts power to the motors to maintain the correct position and speed during movement. With this setup a feedback loop is created to ensure accurate performance. The optical encoders produce 4,000 steps per revolution, and approximately 80,000 counts for the full distance of plunger travel. Therefore control on the microliter scale is possible. The positions are measured and corrected every 0.5 milliseconds enabling motor control on the millisecond time scale.

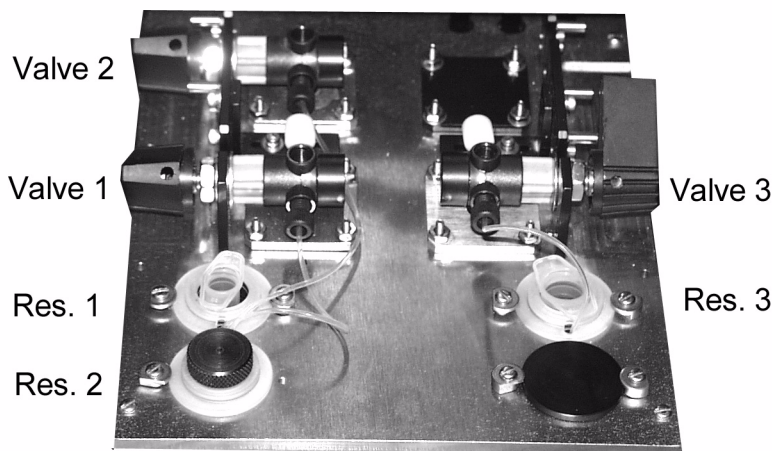
Each motor has its own feedback loop and programmed motion profile. This arrangement gives independent control over each syringe so that any combination of flow ratios or total flow is possible within the limits of the motor's performance. A motor can cover slightly over one magnitude of speed range, so variations in fluid rates and hence dilution ratios can be achieved. Larger variations can be achieved by changing the syringe sizes. The available sizes are one milliliter, two milliliter and five milliliter. Changing the syringes is described below, in the maintenance section.

The syringes are housed in a thermostated block. The block contains Peltier elements with water cooled heat sinks to remove waste heat. There are three temperature probes: one next to the syringes (used for feedback control), one

next to the reservoirs and one attached to a heat sink. The Thermoelectric Power Supply recognizes which thermoelectric device is attached at startup and uses the appropriate tuning constants. Power is delivered to the Peltiers to drive the syringes and Cell Holder to the temperature entered in the user software.

On the top of the syringe drive are the reservoirs and valves. The reservoirs can hold up to 11 milliliters. Samples and buffers can be

loaded into the reservoirs directly or two and five milliliter disposable slip-luer syringes can be slid into the reservoirs so that a seal is made in the base. Using disposable syringes makes cleaning easier and reduces the possibility of cross-contamination. A luer-lock syringe can be made to fit by cutting off the locking sleeve, leaving only the nipple. Caps are supplied to cover the reservoirs when they are not used.



**Figure 9.3:** Stopped-Flow Valves and Reservoirs

The valves direct the flow of liquids. The syringes sit directly beneath the valves and are always connected. The valves switch between the reservoirs (handles in the back positions, flow through the front ports) and the mixer (handles in the front positions, flow through the top ports). When filling and purging the valves should be switched to the reservoir (back) positions. When the mixer and optical cell assembly are being flushed or experimental shots are made the handles should be switched to the mixer (front) positions.

### C. Mixer and Optical Cell

The flows from the valves are sent to the mixer and optical cell assembly. It is here that the solutions are rapidly mixed and sent through the optical cell. A selection of mixers are available: "T", "Fersht" and "Delta". The selection is available so that the mixing and dead time can

be optimized to the sample. The dead time is the time between when the fluid flow has stopped and good quality kinetics data can be measured. The "theoretical dead time" is the volume of sample between the mixing and observation points, divided by the flow rate. Obviously the smaller the volume the better (shorter) the dead time. The shortest dead time is supplied by the "T" mixer, but this also does the least mixing. Highly concentrated samples may not be completely mixed resulting in bad kinetic data. The "Fersht" mixer (named after Dr. Alan Fersht) does an improved job of mixing, but still does not handle the highest concentrations. The "Delta" mixer does the best job of mixing (it will handle saturated guanidine and urea solutions) but has the

largest volume and hence the longest dead time. Below is a table showing the performance that can be expected.

**Table 9.1: Stopped-Flow Mixer Capabilities**

Component	Volume (μl)	Total Volume (μl)	Dead Time (ms)*	Maximum [Urea] (M)
"T" Mixer	1	14	4.6	1
"Fersht" Mixer	3	16	5.3	5
"Delta" Mixer	16	29	9.7	Saturated
Optical Cell	13	-	-	-

\* Theoretical Dead Times at 3 ml/sec. total flow rate.

If the theoretical dead time were the only influence then the shortest dead time would be achieved by running at the highest flow rate. But the actual measured dead time is the time the solution travels between the mixing and observation points, starting when the motors stop to when the signal is stable enough for measurements. Factors that affect the actual dead time are the deceleration of the motors, depressurizing of the system (which keeps the flow moving after the stop), sample properties that affect the mixing (viscosity and density), and the electronic response times (controlled through the software). Deceleration of the motors and depressurizing take longer with faster flow rates, so very high flow rates actually have longer dead times. The compromise between these affects and the theoretical dead time results in an optimal flow rate of 2.5 - 3.0 ml/sec.

After the mixer the solution flows through the optical cell, through a check valve and out into the waste syringe. The optical path length of cell is 1 mm, the width is 2.2 mm. The beam height is ~3 mm so the volume optically sampled is ~6 μl. The check valve isolates the sample from old fluids. The cap screw that sits on the check valve has a standard 1/4 inch diameter, 28 threads to the inch that can accept other chromatography fittings if desired. A diagram that shows the various parts is located below, in the section on Cleaning and Maintenance.

## 9.5 Installing the Stopped-Flow in the Model 202 CD Spectrometer

The stopped-flow accessory can be moved into and out of the sample compartment for rapid kinetic measurements or steady-state types of experiments. To install the stopped-flow:

### A. Remove the existing cell holder

- 1) **Quit instrument control software** if it is running.
- 2) **Turn off the Thermoelectric Power Supply.**
- 3) **Unplug the water lines** to the front panel of the cell holder by pushing the steel buttons and pulling off. They are self sealing and will only release a few drips of water.
- 5) **Unplug the nitrogen line** from the front panel in the same fashion as the water lines.
- 6) **Disconnect the power cables** to the front panel of the cell holder. These have a locking ring near the base that must be unscrewed before the cable can be pulled off.
- 7) **Unscrew the four screws** on the corners of the front panel.
- 8) **Pull the front panel and cell holder away** from the instrument. It rests on rollers in the sample compartment. Be prepared to support the unit when it comes off the last set of rollers.
- 9) **Remove the bottom plate** from the sample compartment. Unscrew the six thumb screws and save them with the cover.

### B. Raise the Stopped-Flow Drive

1) **Remove the two bolts** anchoring the stopped-flow drive to the instrument's frame. These are located on the side walls of the drive near the bottom. The right-angle brackets may need to be loosened so that the drive does not bind between them.

2) **Position the tubing and cables** on the top of the stopped-flow drive away from the edges. As the drive is raised these can become pinched between the drive and edges of the sample compartment.

3) **Turn the hydraulic crank** clock-wise to raise the stopped-flow drive. Raise the drive so that the top is roughly 3 cm above the bottom of the sample compartment.

4) **Connect the power cable** between the top of the Stopped-Flow Tower and the Cell Holder.

5) **Replace the power cable** between the Thermoelectric Power Supply and Stopped-Flow Drive. Remove the cable used for the original cell holder and install the cable specific for the stopped-flow.

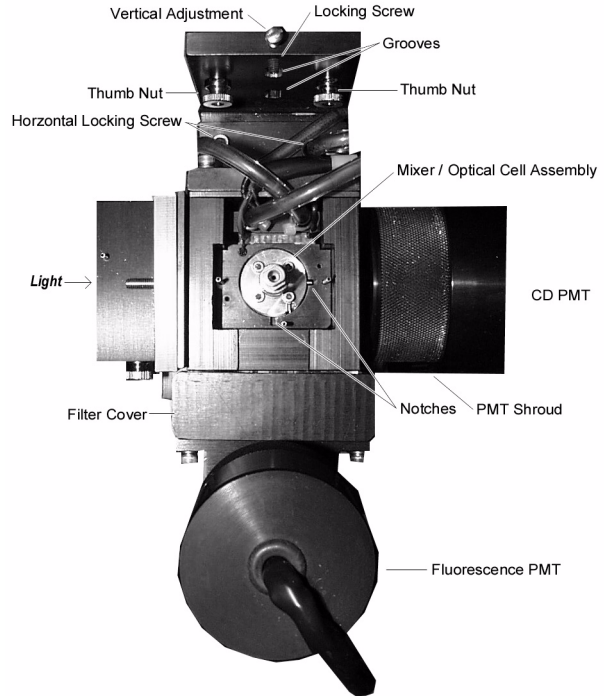
6) **Connect the cables** between the Stopped-Flow Power Supply and the Syringe Drive Tower.

7) **Slide over the Syringe Drive Tower the Light Trap**. The Light Trap rests on the bottom of the sample compartment and fits around the Tower to prevent light from entering the sample compartment. It rests on the bottom of the sample compartment. Tubing and cables should not pass beneath it.

### C. Mount the Cell Holder to the Sample Compartment

1) **Remove from the back plate of the sample compartment two Thumb Nuts**. These are on two screws protruding from the center of the back plate.

2) **Slide the Cell Holder into place**. Position the Cell Holder in front of the Sample Compartment then slide it back into place. It may be necessary to fold the nitrogen tubing (clear tubing) out of the way. If the space is not wide enough to accept the cell holder then screw



**Figure 9.4:** Top View of Stopped-Flow Cell Holder

the PMT Shroud on the right into its mount. The notches on the back wall of the cell holder go over the screws and two pins in the sample compartment's back wall. The screw passing through the top of the cell holder's back mounting plate should rest on the top pin.

3) **Attach the two Thumb Nuts** to the screws snugly to hold the cell holder in place.

4) **Connect the electrical, water and nitrogen lines**. The electrical connector is for the temperature control and is of the same fashion as other electrical connectors. The nitrogen tubing (black) plugs into the nitrogen line entering through the bottom of the sample compartment. The electrical and water lines connect to the lines coming in through the top of the Stopped-Flow Drive.

5) **Connect the cable to the Fluorescence PMT**. This cable emerges from the left side of the sample compartment. Store the terminator formerly attached to the cable in a safe location.

#### D. Check the Optical Alignment

1) **Start the lamp** in the usual manner: purge the instrument with nitrogen; shutdown the computer and turn off the CPU and Instrument power switch; turn on the Lamp Power and Cooling System switch; fire the lamp. Leave the other electronics off.

2) **The light beam should be visible.** Insert the Alignment Target so that the flat surface faces the light. The Target has a pin in its side that should drop into the Side Notch of the Cell Holder.

3) **The light should be centered** around the hole in the target. If not then the cell holder's position will have to be adjusted.

4) **Vertically adjust the cell holder** by slightly loosening the two Thumb Nuts that hold the cell holder to the back wall. Loosen the Locking Screw that holds the Vertical Adjustment screw in place. Turn the Vertical Adjustment screw to raise and lower the cell holder. When in place tighten down the Locking Screw and tighten the two Thumb Nuts.

5) **Horizontally adjust the cell holder** by slightly loosening the two Horizontal Locking Screws. Move the Cell Holder forward or back by hand until the light is centered around the target's hole. Tighten the two Horizontal Locking Screws.

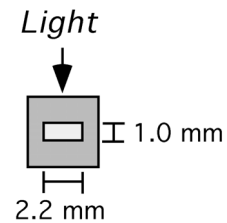
6) **Screw out the shroud covering the PMT** (on the right) so it makes contact with the cell holder. This blocks out light and reduces the amount of nitrogen needed to purge with.

#### E. Mount the Mixer / Optical Cell in Cell Holder

If the Mixer / Optical Cell Assembly is already mounted in the cell holder then this operation can be skipped.

1) **Determine the orientation of the optical cell** in the assembly. The optical cell cross section is 1 by 2.2 mm. The 2.2 mm surface should be facing the beam so that the optical path length is 1 mm. Hold the cell up so that light can be seen through the window. The 1 mm path length will have a clear view while in

the other orientation the walls of the cuvette will faintly seen. The view can be enhanced if air (not water) is in the optical cell. The side showing the walls should be towards the front of the instrument so that the instrument's light will pass entirely through the sample. Note the orientation of the cell relative to the pin on the side of the assembly.



**Figure 9.5: Optical Cell**

2) **Slide the Mixer / Optical Cell Assembly into the Cell Holder.** The assembly has an orientation pin that drops into a notch in the top of the cell holder. There are two notches for the two possible orientations of the assembly. Be sure that the pin is in the notch so the light passes through the 1 mm path length of the cell, with the 2.2 mm surface facing the light.

3) **Attach the assembly cover.** Slide the black cover over the mixer / optical cell assembly, aligning the notches with the black tubing. Use a 5/64" hex wrench to fasten the screws.

4) **Attach the waste syringe.** This is a 25 ml disposable syringe that screws onto the luer lock adaptor in the top of the mixer / optical cell assembly.

#### F. Attach the Mixer's Tubing to the Valves

1) **Fasten the green tubing** from the mixer / cell assembly into the tops of the valves. They should screw down thumb tight. If the mixer is a single stage mixer then the two fluid paths are symmetric and can be connected to any valve. If two or more stages of mixing are used then the fluid paths are not symmetric and the appropriate tubing should be

connected to the right valve. If the valves are not at the right height then the stopped-flow drive can be adjusted with the hydraulic lift.

### G. Finishing Touches

- 1) **Turn on** Thermoelectric Power and Stopped-Flow Supplies. These will not be activated until after the control software has been started.
- 2) **Attach the front plate** to the sample compartment. This plate is similar to the front plate of the thermoelectric cell holder, only without the electrical, water, or mechanical connections.
- 3) **Store the bottom plate** of the sample compartment it's with screws.
- 4) **Start the user interface software.** It will automatically recognize that the stopped-flow is in place. Answer Yes to the initialization question about downloading stopped-flow software. The temperature calibration constants will be automatically selected for the stopped-flow.
- 5) It will be necessary to spend extra time and water purging air from the stopped-flow as the connections have been exposed.

## 9.6 Running Stopped - Flow Experiments

### A. Optimizing Conditions

For optimal performance it is recommended that the one ml syringe be used to inject the smaller volume and the two milliliter syringe be used for the larger injection. For example, the sample is to be diluted in buffer at a ratio of 1:10. In this case the sample should be put in the one milliliter syringe and the buffer placed in the two milliliter syringe. If larger dilution ratios are needed, or a large number of repeat shots are required, then the five milliliter syringe should be used instead of the two milliliter syringe. Likewise the optical mixer should be selected. The appropriate mixer should first ensure complete mixing, then be selected for

the shortest dead time. Changing the syringes and mixer are explained in the Cleaning and Maintenance section.

The dilution ratio has to be calculated to deliver the right concentrations to the flow cell given the initial concentrations of reagents. The total flow rate should be in the range of 2.5 to 3.0 ml/sec. total flow. The dilution ratio and flow rate are determined by the volumes and times entered in the stopped-flow experiment configuration panel (described in the software section of this manual). The final concentration has to be in the range so it is concentrated high enough that a signal can be seen yet the concentration still allow light to pass through. The optimal absorbance is in the range of 0.8 OD at the wavelength being measured.

### B. Loading Solutions

Before the sample and buffers can be loaded the stopped-flow should first be purged of air bubbles. Bubbles have several undesirable effects: they cause the light to be scattered resulting in ruined measurements; bubbles are highly compressible, resulting in delayed fluid flow at the start of the push and delayed stoppage at the end of the push; and the excess compressibility cause mismatched flow rates resulting in the dilution ratio being wrong. To make purging easier and reduce the likelihood of bubbles all solutions should be degassed. This can be done by putting the solutions in a bell jar connected to a water aspirator for 15 minutes. Degassing solutions will help bubbles in the stopped-flow to dissolve back into solution. Degassing also reduces the likelihood that cavitation of the solutions in the mixer results in bubbles. If the stopped-flow has not been used in the same day it probably is best to purge using degassed water first, then replace the water with the buffers and samples once all bubbles have been removed.

To load buffers and samples one of two different strategies can be used. If the solution is abundant and inexpensive (buffers, for example) then it can be used to rinse out the

water. Go to the Control Panels: Syringe Control: Stopped-Flow Syringe Control panel. Change the Set Volume value to 100  $\mu\text{l}$  (press Enter for the new volume to take effect). Switch the valve to the fill position (handle back). Go to the menu for the syringe to be filled and choose the option to Fill Syringe. Switch the valve to the mixer position (handle forward) and in the syringe menu choose Empty Syringe. Repeat this process several times until no bubbles are seen rising in the waste syringe. Afterwards switch the valve to the fill position (handle forward) and in the syringe menu select Purge Syringe. This will partially fill the syringe then quickly empty it. Repeat this process until no bubbles are seen rising in the reservoir or in the tubing connecting the reservoir to the valve. Afterwards change the Set Volume to the syringe's maximum value and use Fill Syringe to load the solution. Switch the valve to the mixer position.

If the solution to be loaded scarce then it can not be used to rinse the lines. It will be necessary to dilute the solution with the water remaining in the syringe and tubing. In principle the water could be removed before the sample is solution is loaded, but removing the water requires removing the syringes to dry them and blowing the tubing with gas to remove the water, steps that are troublesome to perform. Afterwards the sample must be used to remove bubbles, a process that requires more handling. In practice it easier to first purge with water, then slightly dilute the sample and determine the dilution if the concentration is important. All of the water should be removed from the reservoir before the sample is loaded. Next use Purge Syringe to flush the bubbles from the lines and mix the solution. Use Fill Syringe to load the solution in the syringe. It is not necessary to completely fill the syringe. If the solution volume is less than the syringe volume then Set Volume can be used to entirely load the solution without drawing bubbles into the syringe. It is now necessary to fill the tubing between the valve

and mixer. Switch the valve to the mixer (handle forward). Reduce the Set Volume value by 100  $\mu\text{l}$  (press Enter) and choose Fill Syringe. This will push 100  $\mu\text{l}$  into the tubing.

After the solutions are loaded their descriptions should be entered in the Stopped-Flow experiment configuration panel. This information becomes part of the data files.

### C. Making Measurements

After the solutions have been loaded it is necessary to set the experiment parameters in the software. This is also explained in the software section of this manual. Go to the Configure Experiment panel and under Experiment Configuration choose Stopped-Flow Configuration. This panel has the experiment divided into nine time segments, each with it's own time, volume for each syringe to deliver, and number of data points to collect. The flow rates and is determined by the ratio of the volumes to the times, the dilution ratio by the ratio of the volumes and the data collection rates by the ratio of the number of data points to the times. Sometimes it is desirable to measure the signals before the syringes are moved (pre-push). This can be done by entering zero for the volumes and typically 40 milliseconds for Time Segment 1. The second time segment can be used for the push. Typical values for this are for a 30 millisecond push with total volume of 90 microliters. This gives a flow rate of 3.0 milliliters per second. The total volume is split between the syringes to achieve the desired dilution ratio. For a single push the subsequent time segments will have zero for their volumes.

The number of data points specified for each time segment determines the data collection rate and total number of data points. The limits are 10,000 data points per time segment and no faster than 10 microseconds per data point. During the pre-push and push a collection rate of 1 data point per millisecond is adequate. In the time domain following the push the same collection rate should be maintained to capture



**Stopped Flow Configuration** [X]

Stopped Flow Parameters

Time Segment:	1	2	3	4	5	6	7	8	9	Totals	Syr. Capacity
Time	40	40	100	1000	10000	0	0	0	0	0	ms
Syringe # 1	0.000	10.00	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.0000	2000.0 ul
Syringe # 2	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.0000	5000.0 ul
Syringe # 3	0.000	90.00	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.0000	1000.0 ul
Syringe # 4										0.0000	0.0000 ul
Data Points	40	40	100	100	100	0	0	0	0	380	

Syringe #1 : Sample                      Syringe #3 : Buffer  
Syringe #2 : Not used                      Syringe #4 :

Number of Runs : 8                       

**Figure 9.6:** Stopped-Flow Configuration Panel

fast kinetic signals, for example 100 data points for the next 100 milliseconds. Subsequent time domains should be made longer to collect the slower kinetics. The data collection rate can similarly be slowed down; for example, 100 data points for 1000 milliseconds (1 data point per 10 milliseconds) then 100 data points for 10,000 milliseconds (1 data point per 100 milliseconds). The hardware always collects data at the highest possible rate then the software averages the signals to produce a data point. As a result the slower data collection rates have superior noise levels due to the increased averaging. This approach allows fast signals to be collected during and immediately after the push while having excellent noise levels later to measure small signal changes.

The solution descriptions are shown in the Stopped-Flow Configuration panel (as entered by the user in the Stopped-Flow Syringe Control panel). The Number of Runs sets the

number of shots that will be made as part of a single experiment. Initially this should be set to one until good data is being collected. After the instrument is delivering quality data the Number of Runs can be increased to automatically collect a series of shots. Before the stopped-flow experiment parameters can be activated the Configure button must be pushed. This performs a series of checks to make sure the experiment parameters are valid. Checks are made of the minimum and maximum flow rates, the number and rate of data points collected, and other items. If one of the checks fail then the user should make the appropriate changes. Some checks will prevent the experiment from being done while other checks will allow experiment although the instrument is operating beyond its calibrated range. If both the user and software are satisfied then the OK button becomes available. Pushing OK accepts the experiment

parameters, closes the panel and makes Stopped-Flow experiments the current experiment.

The Configure Experiment parameters also need to be set. The Time Constant normally defaults to 100 milliseconds for experiments other than stopped-flow. When stopped-flow experiments are configured (by the Exit / Save Configuration menu item) the default switches to 100 microseconds so that fast kinetics can be measured. If only slow kinetics are to be measured then the Time Constant can be made slower to filter out more noise. The Time Constant should be more than three times faster than the fastest kinetics to be measured otherwise the instrument's response time will distort the signal. The Time Constant switches to an experiment's default when ever the experiment type is changed, so customizing the Time Constant requires returning to the Configure Experiment panel after the experiment type has been set.

Use Save Data Options to select the types of data to be saved. In stopped-flow experiment mode the Dynode high voltage is static so recording it is of little use. In CD instruments the CD Current (Abs) signal will change linearly with the sample's percent transmission, so it can be saved to follow absorbance changes. An equation exists in the Math Operations menu to convert this signal to absorbance. The X-axis for stopped-flow experiments is time, so it is not necessary to record the Elapsed Time. Turn on the Fluorescence and QC Signals if these are desired. Leave the panel using OK for the selections to take effect.

While in the Configure Experiment panel the wavelength and bandwidth should be set for the stopped-flow experiment. If the temperature is changed then the user should wait until after the drive and mixer have equilibrated to the new temperature. Detailed temperature information is available through the main panel's Control Panel: Temperature Control

menu. The Description, Name and Experiment Counter can be updated as well. In fluorometers the dark correction based on a single dark measurement at the start of the experiment is possible, but correction based on dark measurements for each data point is not possible.

At this point in time the optical cell does not contain the correct concentration of diluted sample, so it is not practical to set the PMT high voltages yet. Leave the Configure Experiment panel with Exit / Save Configuration. Make a final check to make sure the valves are in the correct positions. Leave the covers off the sample compartment and Press Run Experiment to make a single shot. Watch the waste syringe to make that no bubbles emerge from the Mixer / Optical Cell assembly. Repeat making shots until no bubbles are seen. Replace the covers on the sample compartment.

By this time the line and mixer are purged and approximately the correct concentration of sample is in the optical cell. Go to the Control Panels: PMT Control menu. In fluorometers the fluorescence and quantum correction measurements are made with the PMT high voltages held constant. This remains true for stopped-flow experiments. Set the high voltage for these detectors as described in the software chapter.

CD measurements are different matter though. In experiment modes besides stopped-flow the CD PMT dynode high voltage is automatically adjusted to maintain a constant current passing through the PMT. In stopped-flow experiment mode the signals change too fast for the high voltage servo, so in this experiment mode only the dynode high voltage is locked and the current is allowed to change. This requires setting the dynode high voltage manually to the correct value (close to 1.0). Deviations from this value can result in calibration errors. Pressing Autoset under CD-PMT Values will automatically ramp the dynode high

voltage until a current value close to 1 is achieved. If this does not happen then pressing AutoSet a second time usually finishes the job. Manually setting the Dynode can be used for fine adjustments if necessary. The Input Range need not be changed. Fluorescence measurements can be made simultaneously with the CD measurement if the fluorescence accessory is available. If the CD instrument is equipped with the Fluorescence Detected CD option (FD CD) then the user will most likely want the CD Signal Source to be CD. The Fluorescence Measurement Excitation Wavelength should be made to match the current wavelength. FI Averaging Time does not matter because the CD and fluorescence signals are measured simultaneously. Press Auto Set at Excitation WL to set the fluorescence PMT's dynode high voltage. If the fluorescence signal makes large changes the HV Setpoint can be manually changed to make better use of the signal's full scale (0 - 5). If the quantum counter (QC) accessory is present then its high voltage should be set as well. Press Auto Set to turn it on. The QC signal can be used to automatically correct the "absorbance" and fluorescence signals by choosing the Apply QC Correction check box. Press Return to go back to the main panel.

At this point it is desirable to make a few more single stopped-flow shots to ensure the right sample concentration in the optical cell. Changes in the concentration can be followed by the optical signals reported in the main panel. If the values migrate from the original values then the high voltages should be adjusted in the PMT Control panel. Once these parameters have stabilized then the instrument is ready to collect data. If a single shot does not provide data with sufficient signal-to-noise ratio then it will be necessary to average multiple shots together. Data from different experiments can be averaged together by displaying the data in Data Review and using the Axis Definitions: Data Review Average (see software portion of this manual for a description). A

single experiment can also be programmed to make multiple shots by changing the stopped-flow experiment configuration. The multiple shots can then be averaged using the Axis Definitions: Data Collection Average menu.

Saving data is done in the same fashion as other experiment types, except the default file format is binary. Binary files are smaller and move more quickly than text files, a desirable feature for data rich stopped-flow experiments. If all of the data points possible were collected in a stopped-flow experiment and all of the data types were turned on then the result is over 1 million numbers. If then multiple shots are made in a single experiment then the data file becomes even larger. Stopped-flow experiment can be manually saved to text in the Data Browser by using the Export Data Set: Export to ASCII Text option. The file format is the same as used for other experiment types. If a single data set is selected then only it and the time are saved to the same text file format. Probably the best approach is to save all of the raw data to a binary file as soon as the experiment is done, then later save the averaged and other calculated data to text files for exporting to other programs.

## 9.7 Cleaning and Maintenance

Good cleaning of the instrument is important for it to continue collecting quality data. Cleaning should be done in the same manner as for regular cuvettes. First the solutions should be pushed back into their reservoirs by switching the valves to fill positions and choosing Empty Syringe in the Syringe Control panel. Remove the solutions and replace them with distilled water. Use the Purge option to rinse out the syringes. Replace the water with fresh water and fill the syringes. Switch the valves to the mixer positions and empty the syringes through the mixer / optical cell assembly. Empty the waste syringe and repeat this process several more times. This cleaning is sufficient for salt solutions, but sticky substances require further cleaning. Deter-

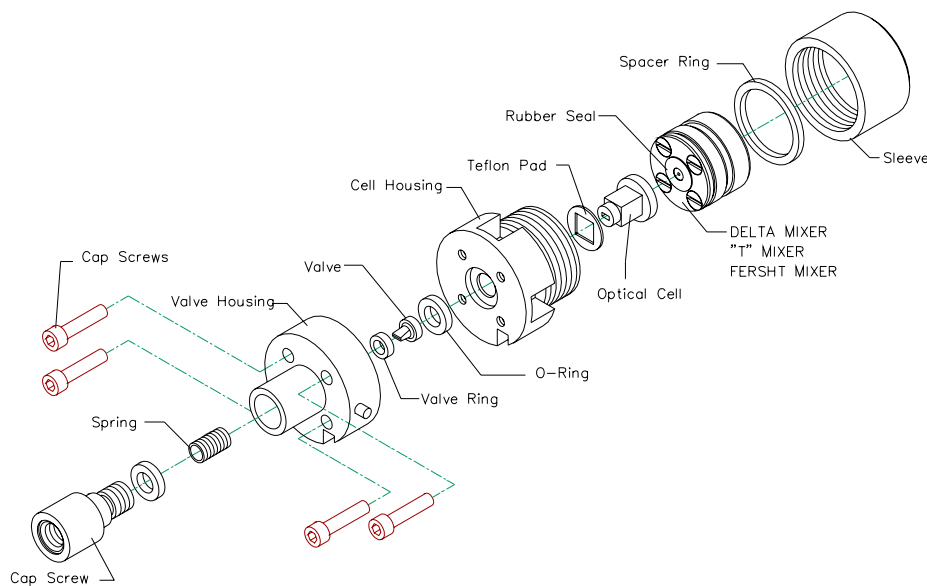
gents can be used, although SDS should be avoided (it forms insoluble precipitate when mixed with potassium ions). Commercially available detergents or Tween are good choices. Afterwards the system should be rinsed with water to remove the bulk of the detergent, ethanol to remove detergent on the surfaces, then more water. If the stopped-flow is to be used in the next week then it can be stored with water in the lines. If it is to be stored for longer periods then it should be filled with a 10% ethanol solution.

It may be necessary to clean the system more severely than is possible by rinsing through solutions. For example, proteins may stick to the optical cell, causing poor optical baselines

or high light absorption. Fluid lines may clog if samples contain particulates. In these cases it is necessary to disassembly the mixer / optical cell assembly and individually clean the parts. To do this:

#### A. Remove the Mixer / Optical Cell Assembly

- 1) **Unscrew the fluid lines** from the tops of the valves
- 2) **Unscrew the waste syringe** from the top of the assembly
- 3) **Remove the cap** covering the assembly by loosening the two screws (use a 5/64" hex wrench) and lift off the cap.
- 4) **Lift the mixer / optical cell assembly** out of it's holder.



**Figure 9.7: Mixer and Optical Cell Assembly**

#### B. Disassemble the Assembly

- 1) **Remove the mixer** by unscrewing the Sleeve that threads onto the bottom of the assembly. Slide the mixer out. If the mixer is tight then grip the base of the tubing and rock the mixer while pulling out.

- 2) **Remove the Cap Screw** from the top of the assembly
- 3) From this point forward there is a risk of the optical cell unexpectedly separating from the assembly. Either place your hand or some soft material underneath for the cell to fall onto.

Use a 3/32" hex wrench to **remove the four bolts** from the top of the assembly. The Valve Housing and Cell Housing are now separate.

4) The Spring, Valve and Valve Ring are now loose. **Remove the optical cell** by pushing on the it's top in the center of the O-Ring.

#### C. Disassemble the Mixer (optional)

1) **Remove the four screws** in the mixer's top. The order and orientation of the layers are important. As the unit is taken apart note the details.

2) **Remove the tubing** from the base with the miniature wrench supplied with the stopped-flow.

#### D. Clean the Parts

1) Use standard laboratory cleaning procedures for cleaning the parts. A bath (not probe) sonicator can be used to speed the process. The optical cell is made of quartz and can be etched by pH's greater than 10. It is immune to low pH's so cleaning it with acids is acceptable.

#### E. Reassemble the Assembly

1) **Reassemble the mixer** in the reverse order it was taken apart. Leave the tubing off until later. Tighten down the screws hand hard.

2) **Insert the optical cell** into the Cell Housing. Be sure the Teflon Pad is in place over the Optical Cell. Keep the assembly in it's side to keep the cell from falling out.

3) **Place the Rubber Seal** in the center of the top of the mixer. Tilt the Cell Housing and slide the mixer into housing. **Screw on the Sleeve** to hold the Mixer and Optical Cell in place. The sleeve should be snug but not screwed on hard.

4) **Place the O-Ring** over the top of the Optical Cell in the Cell Housing.

5) **Put the Valve Ring over the Valve and insert both into the bottom of the Valve Housing.** Hold the Cell Housing on its side and put the Valve Housing in position so that the groove in the sides of the two parts is aligned. **Screw the four bolts in** to fasten the two housings together.

6) **Place the spring into the top of the valve housing. Screw the Cap Screw** down all of the way.

7) **Screw the tubing on loosely** to the base of the mixer by hand. Use the miniature wrench to attache the tubing. Use the miniature torque wrench to tighten down the fittings. The torque wrench has two arms: a shorter, thinner one labeled "5 ozf-in" and a longer, thicker are labeled "10 ozf-in". When one arm is pushed so that it barely touches the other arm then that amount of torque is being applied. The fittings should be tightened with a minimum of 5 ounce-inches, but no more than 10 once-inches. Over tightening will damage the fittings resulting in leaks.

8) **Mount the assembly** using the procedures given earlier in "Installing the Stopped-Flow".

## 9.8 Changing Mixers

The mixers may be changed to optimize the performance for the sample's characteristics. The necessary instructions are in section entitled Cleaning and Maintenance. The subsections describing the valve and optical cell can be skipped.

## 9.9 Changing Syringes

Normally it is not necessary to remove the syringes: they can be cleaned in place. Occasionally the syringes may be removed to inspect their conditions or exchange for a different size.

#### A. To Remove a Syringe

1) **Quit the software** if it is running.

2) **Turn off power** to the stopped-flow motor power supply.

3) **Remove the front, middle plate** of the stopped-flow tower. It is attached with two thumb screws. It may be necessary to remove the tubing from the tops of the valves and use the hydraulic jack to lower the stopped-flow drive to a more accessible position.

4) Syringes one and three are accessible

from the front. Syringes two and four sit behind one and three. To service syringes two and four it is easiest to detach the plunger from syringes one and three by unscrewing the caps and pushing their plungers up into the syringes to make room.

5) **Detach the plunger from the drive.** Unscrew the cap that attaches the plunger and slide it from around the plunger. Push the plunger up into the syringe.

6) **Push the drive down to nearly the bottom.** It will require some weight to force the drive to move downward. If the drive is too stiff to push then turn the lead screw by hand to move the drive down. Try not to push it all of the way down.

7) **Loosen the two thumb screws** next to the syringe. Swing the locking cams away from the syringe.

8) **Unscrew the syringe.** When it is free it will drop down.

#### **B. To Install a Syringe**

1) Follow steps 1 - 4 under "To Remove a Syringe" if they have not already been done.

2) **Screw the syringe up** into the stopped-flow. The locking cams may move into the wrong position and impede progress. The syringe should be screwed in until it is hand tight.

3) **Swing the locking cams into position** beside the syringe and tighten the thumb nuts. The locking cams prevent the syringe from backing out.

4) **Raise the drive** so to within 5 cm of the plunger. If the drive can not be raised then the lead screw can be turned by hand to raise the drive.

5) **Pull the plunger down** into the top of the drive and slide the cap around the plunger. Screw down the cap firmly to fasten the plunger to the drive.

6) **Replace the middle cover** on the stopped-flow tower. **Turn on power** to stopped-flow motor power supply.

7) If the syringe is the same one as earlier

removed then the unit is ready to use. If not then it will necessary to recalibrate the syringe drive. Calibration instructions are given below.

### **9.10 Calibration of Syringe Drive**

The motion of the plungers, and hence the flow rates and dilution ratios, is based on a feed-back loop. This loop measures the motor speeds and adjusts power to the motors to achieve the correct speeds. This loop relies on several tuning parameters that convert the motion information to the appropriate power levels. If the mechanical loading of the system changes then it will be necessary to recalibrate the stopped-flow. Examples of when calibration should be done are if the syringe size has been changed, the unit has been used extensively and wear has changed the performance, or the mixers have been changed.

Calibration is done with the syringe utility program SCT.EXE (Syringe Control Terminal). The name may be slightly different if the version number has been added to the file-name. When SCT is started it asks "Do you want to reload existing calibration data?" Answer "Yes". The program performs several functions related to the stopped-flow drive, one of which is calibration. **Syringe Motor Control** turns the power **On** and **Off** in the stopped-flow power supply. The **Syringe Control Panel** is the same as in the user programs. Under **Options** the item **Syringe Status** shows the absolute positions of the motors and the limits switches, and **Service Panel** gives the software version number.

**Calibrate Axis** contains the options necessary to calibrate the stopped-flow. **Calibrate Syringe #1, #2 and #3** starts calibration of those individual syringes while **Stop Current Calibration** aborts the process. **Constant Volume Calibration** starts a process where two syringes are moved simultaneously to maintain the same flow rate through out the calibration. The advantage of this approach is that the hydraulic back pressure is the same as

during actual experiments yielding more accurate calibration values. A second (reference) syringe is used to deliver the additional volume needed to maintain the selected flow rate. This approach depends on the calibration of the reference syringe. **Display Calibration Results** shows the values obtained for each syringe shot while **Display Calculated Results** shows the calibration values obtained for each syringe speed. **Write FV Data File** saves the calibration values to file.

#### A. Before Calibration

To calibrate the stopped-flow must first be assembled in the configuration that it will be used in, then it must be filled and purged with freshly degassed water. This is done in the same manner as in the user software, with the exception that the motor driver software must be manually loaded.

- 1) Go to the **Syringe Control** panel and under the **Syringe Drive Control** menu select **Init and Reload Syringe Drive Controller**. The stopped-flow power supply will turn on.
- 2) If the syringe size(s) have been changed then use **Change Syringe Size** for the individual syringes. Next **Home**, **Purge** and **Fill** the same as always. When all of the bubbles have been removed leave the Syringe Control panel.

#### B. Single Syringe Calibration

- 1) **Select the syringe in the Calibrate Axis menu.**
- 2) The user is prompted to **move the valves at the appropriate times**. The program starts by moving the syringe at the slowest speed (5000 Counts Per Second) and measures the error in the speed. Ideally the graph will show a straight line, but in reality acceleration and deceleration errors will appear. The feedback values are adjusted to minimize the error during the steady-state portion of the movement. Once the appropriate feedback value is found the speed is raised and the optimization process is repeated.
- 3) The user is prompted to **move the valves**

**to refill the syringes** at the appropriate times.

4) The speed is increased until either it reaches 80,000 CPS, the user stops the calibration, or the steady-state speed can not be maintained. When one of these conditions is reached the **user is asked if the calibration values should be saved**. Answer Yes. The calibration values and the range of speeds covered is saved to the calibration file. This information is automatically read every time the user software is started.

#### C. Constant Volume Calibration

- 1) **Select the Reference syringe.** This syringe should be calibrated prior to using the Constant Volume Calibration, either by calibrating it individually, or by using another calibrated syringe as the reference.
- 2) **The Flow Rate must also be selected** to be close to experiment conditions. For stopped-flow drives without electric valves the optimal flow rate is 2.5 - 3.0 ml/sec.
- 3 - 4) **The Constant Volume calibration proceeds the in the same fashion** as calibrating a single syringe. The volume of each syringe is tracked and the user will be prompted to move the appropriate valves when refilling is necessary.

#### D. After Calibration

When the calibration is completed a record of the results can be saved to other file formats beside that used by the user programs. Both Display Calibration Results and Display Calculated Results allows the data to be saved and exported. These are convenient so that a record of the calibration is kept.

### 9.11 Removing the Stopped-Flow from the CD Spectrometer

Before removing the stopped flow the system should be thoroughly cleaned. The system should be left containing distilled water and 10% ethanol.

**A. Remove the Mixer / Flow Cell Assembly**

- 1) **Quit the software. Turn off the power** to the Stopped-Flow Power Supply and Thermo-electric Power Supply.
- 2) **Remove the sample compartment covers** from the front and top.
- 3) **Unscrew the fluid lines** from the tops of the valves
- 4) **Empty and remove the Waste Syringe** from the top of the assembly.
- 5) **Remove the Assembly Cover** over the mixer / flow cell assembly. Use a 5/64 inch hex wrench to loosen the screws.
- 6) **Lift out the Mixer / Flow Cell Assembly** from the cell holder.

**B. Remove the Cell Holder**

- 1) **Disconnect electrical, water and nitrogen lines.** The water and nitrogen lines separate by pushing on the metal tab. They are self-sealing. The electrical lines disconnect by unscrewing the knurled ring then pulling the ends apart.
- 2) **Screw back the PMT shroud.** Screw it back far enough to give a few millimeter clearance.
- 3) **Remove the two thumb nuts** attaching the Cell Holder to the sample compartment's back wall. Be prepared to support the cell holder with one hand.
- 4) **Remove the Cell Holder** by sliding it forward and out of the sample compartment. The tubing and cables will have to be moved to clear the optics.
- 5) **Reattach the thumb nuts** to the back wall and place the cell holder in a safe location.
- 6) **Attach to the fluorescence cable** in the sample compartment the terminator.

**C. Lower the Stopped-Flow Drive**

- 1) **Empty the reservoirs** if not already done.
- 2) **Place the caps on the reservoirs.**
- 3) **Remove the two bolts** anchoring the stopped-flow drive to the frame. These are located on the side walls of the drive near the bottom. The bolts on the right-angle brackets may have to be loosened so the drive does not

bind between them.

- 4) **Turn the hydraulic crank** counter clockwise to lower the drive. Lower it until the electrical connections at the base of the stopped-flow tower nearly touch the table.
- 5) **Remove the light trap** from the sample compartment. This is the square metal plate that sits loose on the bottom of the compartment. Store it in a safe location.
- 6) **Position the tubing and connectors** so that they are out of the sample compartment.
- 7) **Install the bottom plate** in the sample compartment and fasten down with the six thumb screws.

**D. Install the new cell holder**

- 1) The sample compartment sits on the rollers attached to the sides of the sample compartment. **Position the end of the cell holder on the first roller** and push it into the sample compartment.
- 2) **Fasten the cell holder into place** with the four spring screws on the front cover of the cell holder.
- 3) **Detach the two water lines** from the side of the Stopped-Flow Tower. Attach these to the connectors on the front of the cell holder.
- 4) Hanging beneath the sample compartment is an unused nitrogen line. Attach this to the front of the cell holder.
- 5) If the new cell holder has a fluorescence PMT then remove and store the terminator and attached the cable in the sample compartment to the PMT.
- 5) The power cable for each type of cell holder is different. **Attach the appropriate power cable to the front of the cell holder.** Detach from the back of the Thermoelectric Power Supply the power cable that runs to the Stopped-Flow Tower. Attach to the Thermoelectric Power Supply the power cable from the cell holder.
- 6) **Turn on power** to the Thermoelectric Power Supply. Wait at least 15 seconds before starting the software.



7) **Run the user software.** The software will automatically configure itself for the new hardware.



## Use of the Aviv Model 410 Circular Dichroism Spectrometer

Note: this list is to serve as a reminder, and is not a substitute for proper training on the instrument's use.

### Starting the instrument...

- 1) Turn on the **nitrogen purge**. Purge at least 20 minutes prior to turning on the lamp.
- 2) Turn on the lamp:
  - a. ALL other electronics should be turned OFF.
  - b. Turn ON the **XE LAMP PWR** switch.
  - c. Wait for the **LAMP READY** light to come on.
  - d. Push the red **IGNITE LAMP** button.
- 3) Turn ON the **CPU/INST PWR** switch.
- 4) Start the computer.
- 5) Start the **Thermocube** chiller (power switch is on left side).
- 6) Start the instrument software.
  - a. Set up the experiment with **Configure the Experiment**.
  - b. Select the datasets in **Save Data Sets**.
  - c. Select the Data Path in the **Displays: Data Browser**
  - d. Run experiments.
  - e. Perform averaging and math operations as needed.
  - f. Save data via Data Browser

### Turning off the instrument...

- 1) Save any remaining data
- 2) Use **File: Terminate CDS Program** to shut down the software.
  - a. A last opportunity is given to save any data to the hard drive that may not have already been saved.
  - b. The wavelength will move to the home position, causing a delay in the program's termination.
- 3) Turn OFF the **XE LAMP PWR** switch
- 4) Turn OFF the **Thermocube**.
- 5) Wait 10 minutes before turning off the nitrogen flow. Other steps can be done without waiting for the nitrogen flow.
- 6) Clean cuvettes, syringe pump and other accessories if they were used.
- 7) Copy or move data off instrument computer.
- 8) Shut down the **Windows** software.
- 9) Turn OFF the **CPU/INST PWR** switch.