

WINFAC

(Fluorescence Analysis)

Version 1.0

Users Guide

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Getting Started

Introduction

WINFAC collects intensity data from samples containing fluorescent dyes using a Perkin-Elmer LS-50 or LS-50B luminescence spectrofluorimeter. The program provides bookkeeping for multiple samples with one or more fluorescent dyes in each sample. The spectrofluorimeter is controlled through a serial interface by WINFAC running on a PC. WINFAC is public domain software that will evolve to meet the needs of researchers using fluorescent microsphere technology. We encourage users to report bugs and suggest how the program may better meet their needs. This program is maintained and supported by the Fluorescent Microsphere Resource Center. Updates are available through the FMRC web site at <http://fmrc.pulmcc.washington.edu/>.

Hardware Requirements

WINFAC has been tested under the following conditions and we recommend them as a minimum for this program.

- PC fitted with a 486 (66 MHz) processor or higher
- WINDOWS 95 or WINDOWS 98
- At least 16Mb RAM
- One serial communication port to interface with the spectrofluorimeter.

If the autosampler option is employed the following additional hardware is required:

- One serial communication port to interface with the diluter station.
- One serial communication port to interface with the autosampler.

Program Features

WINFAC is an update to FAC8 (menu driven program) that runs under the DOS operating system. Along with a totally new Windows environment interface come additional features as follows:

For fixed wavelength analysis WINFAC now reads the fluorescent intensities of samples at up to 30 different excitation-emission wavelength pairs. Each wavelength pair may differ in any of four settings: 1) excitation wavelength, 2) emission wavelength, 3) excitation slit width, and 4) emission slit width. This flexibility allows the same sample to be read under multiple conditions without user intervention.

Ability to run synchronous scans separately or in conjunction with reading fixed wavelength intensities on each sample. Currently each sample may be analyzed via synchronous scan under only one set of conditions.

Capability to designate pure samples to allow FMSPILL to deconvolute synchronous scan spectra and/or generate spillover matrices from fixed wavelength data for use in matrix inversion spillover correction..

Ability to designate a sample as a control or solvent blank.

Improved interface for creating method and viewing analysis.

Storable method which can be recalled and used during subsequent analyses.

Multiple output options: writes fluorescent intensity values to both screen and text file, with optional printer output.

Watercell time drive to verify that the spectrofluorimeter is functioning properly.

Option of accepting or rejecting the values after all wavelength pairs are gathered. Only accepted samples are written to the text file and printer. There is a comment line that may be used to identify sample irregularities for each sample if analyzing samples via cuvette.

The output to the text file may be formatted for either DOS (tab delimited, carriage return/linefeed) or Macintosh (tab delimited, carriage return).

Program Acquisition

WINFAC is available for download from the web site of the Fluorescent Microsphere Resource Center (<http://fmrc.pulmcc.washington.edu/fmrc/software>). If you are unable to access the web you may request a copy of the program on diskette by contacting the FMRC staff through one of the following means:

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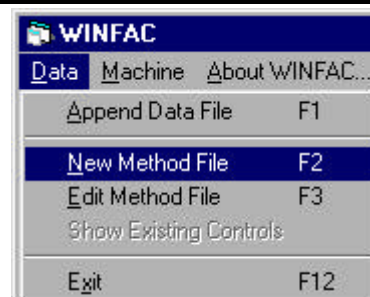
The Method File

Overview

WINFAC uses method files (.MTH) to store parameters needed to setup and drive the spectrofluorimeter. Once created subsequent analyses may be performed without risk of parameter definition error. Once a method is defined for use with one set of experiments it may then be used directly for new experiments or may be changed and resaved to establish a modified set of parameters.

Creating A Method File

To create a new method, select the **New Method File** sub-menu from the **Data** menu. Figure 2-1 shows a newly generated method form with defaults selected. Following are explanations for the parameters required when filling out the form.



The screenshot shows the WINFAC application window with the 'Data' menu open. The main form is titled 'WINFAC' and contains several sections for configuring a method file.

- Scan Type:** Radio buttons for 'Fixed Wavelength' (selected), 'Synchronous', and 'Both'.
- # Colors:** A text box containing '1' and an 'Edit Colors' button.
- Scan Speed:** A text box containing '480' followed by 'nm/min.'.
- Read Time (.1-100 sec):** A text box containing '10.0'.
- Pures:** A text box containing '10.0'.
- Samples:** A text box containing '1.0'.
- Generate Spillover Matrix:** A checked checkbox.
- File Format:** Radio buttons for 'DOS/Windows' (selected) and 'Macintosh'. A 'Print Results' checkbox is also present.
- Delivery Method:** Radio buttons for 'Cuvette' (selected) and 'AutoSampler'.
- PMT Voltage:** Radio buttons for 'Default' and 'Selected' (selected), with a text box containing '820'.
- Emission Filter:** Radio buttons for 'None', '290 nm cutoff', '350 nm cutoff' (selected), '390 nm cutoff', '430 nm cutoff', '530 nm cutoff', and '1% transmittance attenuator'.
- Amount of solvent:** A text box containing '2'.
- Analyst:** An empty text box.
- Sample Id:** An empty text box.
- Starting Number:** A text box containing '1'.
- Buttons:** 'Save As' and 'Cancel' buttons at the bottom right.
- CURRENT FILES:** A section at the bottom showing 'Analysis Parameter File: None open' and 'Analysis Data File: None open'.

Figure 2-1 Method form set for fixed wavelength scanning.

SCAN TYPE

Fixed Wavelength

The excitation/emission wavelengths and slit positions are defined (fixed) for each fluorescent dye to be analyzed. Advantage: fixed wavelength analyses of multiple dyes are fast when compared to synchronous scans. Disadvantages: optimal settings for the excitation and emission wavelengths vary depending upon the number of dye present and analyzed in the sample. In order to determine optimal settings considerable experimentation may be required in order to optimize signal intensities while minimizing spillover from adjacent colors.

The screenshot shows the WINFAC software window with the following settings:

- Scan Type:** Synchronous (selected), Fixed Wavelength, Both.
- # Colors:** 1
- Scan Speed:** 480 nm/min.
- Read Time (.1-100 sec):** (empty)
- Pures:** 10.0
- Samples:** 1.0
- Generate Spillover Matrix:** checked
- PMT Voltage:** Selected 820 (Default, Selected).
- Emission Filter:** 350 nm cutoff (selected), None, 290 nm cutoff, 390 nm cutoff, 430 nm cutoff, 530 nm cutoff, 1% transmittance attenuator.
- File Format:** DOS/Windows (selected), Macintosh.
- Delivery Method:** Cuvette (selected), AutoSampler.
- Print Results:** unchecked
- Amount of solvent:** 2
- Analyst:** (empty)
- Sample Id:** (empty)
- Starting Number:** 1
- Slit Settings:** Excitation (2.5-15): (empty), Emission (2.5-20): (empty).
- Scan Limits (nm):** Lower Limit (Ex): (empty), Upper Limit (Ex): (empty), Offset [Em-Ex]: (empty), Data Interval: (empty).
- CURRENT FILES:** Analysis Parameter File: None open, Analysis Data File: None open.

Figure 2-2 Method form set for synchronous scanning.

Synchronous

Synchronous scans set the excitation and emission wavelengths a specified number of nanometers apart and then both monochromators move synchronously from a starting wavelength along the entire wavelength spectrum until the desired upper limit is reached (fig 2-2). Values are read at specified wavelength increments during the scan and a spectrum generated. Advantages: ease of

use is a hallmark of synchronous scanning since you do not have to specify wavelengths and slit positions for each dye. Disadvantages: even when there is minimal spillover between dyes you must run the spectrum through deconvolution software (FMSPILL) in order to obtain intensity levels for each color. It is also necessary to run pure samples for each experiment in order for FMSPILL to locate each dye peak. This establishes the points on each spectrum to be used in determining dye intensities.

Both

Selects both fixed wavelength and synchronous scan analysis on the same sample (fig 2-3). Fixed wavelength intensities are gathered prior to performing the synchronous scan.

The screenshot shows the WINFAC software window with the following settings:

- Scan Type:** ☒ Fixed Wavelength, ☐ Synchronous, ☒ Both
- PMT Voltage:** ☐ Default, ☒ Selected 820
- Emission Filter:** ☐ None, ☐ 290 nm cutoff, ☒ 350 nm cutoff, ☐ 390 nm cutoff, ☐ 430 nm cutoff, ☐ 530 nm cutoff, ☐ 1% transmittance attenuator
- # Colors:** 1 (with Edit Colors button)
- Scan Speed:** 480 nm/min.
- Read Time (.1-100 sec):** (empty field)
- Pures:** 10.0 **Samples:** 1.0
- ☒ Generate Spillover Matrix
- Synchronous Scan:**
 - Slit Settings:** Excitation (2.5-15): (empty), Emission (2.5-20): (empty)
 - Scan Limits (nm):** Lower Limit (Ex): (empty), Upper Limit (Ex): (empty), Offset [Em-Ex]: (empty), Data Interval: (empty)
- File Format:** ☒ DOS/Windows, ☐ Macintosh
- Delivery Method:** ☒ Cuvette, ☐ AutoSampler
- ☐ Print Results
- Amount of solvent:** 2
- Analyst:** (empty field)
- Sample Id:** (empty field)
- Starting Number:** 1
- Buttons:** Save As, Cancel
- CURRENT FILES:** Analysis Parameter File: None open, Analysis Data File: None open.

Figure 2-3. Method form set for both fixed wavelength and synchronous scanning.

PMT Voltage

This voltage applies to the entire method. The PMT voltage can not be varied according to the color being analyzed or scan type being used.

Default Sets the default voltages which are defined according to the excitation slit position.

Selected Set a specific PMT voltage. Input must be in the range of 0-999 volts.

Emission Filter

Select the emission filter position that applies to the entire analysis utilizing this method. The emission filter may not be varied according to the dye being analyzed or scan type being used.

Colors

Range: Min: 0 Max: 30

Number of wavelength settings to be analyzed by fixed wavelength analysis. To analyze fluorescent dyes under multiple conditions (i.e., differing excitation or emission wavelengths or differing slit positions) indicate the total number of analyses per sample that you desire in this variable. If reading intensity levels for six dyes, but there are two settings per dye, enter 12 in this box. For analyses which require pure samples the number of wavelength pairs must be **exactly** the same number as there are fluorescent dyes in the sample. Pure samples **must be** present if performing synchronous scans (FMSPILL uses them to identify the peaks to analyze as well as set up the microspheres/ml conversions if requested), while pure samples are only required for fixed wavelength analyses if post-processing spillover correction is required.

Colors: [Edit Colors](#)

Scan Speed: nm/min.

Read Time (.1-100 sec)

Pures: Samples:

☒ Generate Spillover Matrix

Edit Colors

This button is available if either **Fixed Wavelength** or **Both** are selected under Scan Type. The form displays edit boxes for all wavelengths if the number of colors is less or equal to 15. Otherwise the form displays the first 15 by default and you must use the **Next 15** or **Prev 15** buttons to navigate between pages of the form.

Fixed Wavelengths					
	Wavelength		Slits		
	Ex	Em	Ex	Em	Color
1	<input type="text" value="430"/>	<input type="text" value="465"/>	<input type="text" value="4"/>	<input type="text" value="4"/>	Blue-Green
2	<input type="text" value="443"/>	<input type="text" value="492"/>	<input type="text" value="4"/>	<input type="text" value="4"/>	Green
3	<input type="text" value="510"/>	<input type="text" value="522"/>	<input type="text" value="5"/>	<input type="text" value="5"/>	Yellow
4	<input type="text" value="610"/>	<input type="text" value="635"/>	<input type="text" value="4"/>	<input type="text" value="4"/>	Crimson

Figure 2-4. Wavelength settings dialog box.

For each dye intensity to be read you must supply an excitation wavelength (Ex), and emission wavelength (Em), an excitation slit width (Ex) and emission slit width (Em) (figure 2-4). Click on the Color popup button and select the dye name of the wavelength pair being analyzed. This

information is saved in both the method and data files to better identify each wavelength pair selected.

All values for both wavelength and slit position entries are rounded to the nearest 0.1 nm.

Excitation Wavelength	Range: 200 - 800 nm
Emission Wavelength	Range: 200 - 900 nm
Excitation Slit Position	Range: 2.5 - 15 nm
Emission Slit Position	Range: 2.5 - 20 nm

Scan Speed Range: 10 to 1500 or -1500 to -10 nm/min

Set the speed at which scans are performed during analyses.

Read Time

- Pures** This setting determines the duration (seconds) over which sampling is averaged and reported for pure samples. Pure samples are separated out to allow for maximal accuracy in determining the spillover matrix without forcing the entire sample set to be read at the enhanced duration.
- Samples** This setting determines the duration (seconds) over which sampling is averaged and reported for regular samples.

Generate Spillover Matrix

Select this option to read pure samples at the start of an analysis in order to provide data to FMSPILL in order to generate a spillover matrix or identify dye peaks. If you quit an analysis while reading pure samples or during the initial standard or blank samples, the program erases the data file in order to force a complete reread of the pure samples. This ensures that all pure colors were read under similar machine conditions. If you quit any time after you read the blank and pure samples you may append to the data file without any further requests for pure color samples. Pure samples are only requested at the beginning of an analysis, not every time a new tray (autosampler) or session (cuvette) is started.

Synchronous Scan Section

This section establishes the parameters for performing a synchronous scan.

Excitation Slit Position	Range: 2.5 - 15 nm
Emission Slit Position	Range: 2.5 - 20 nm

Synchronous Scan

Slit Settings

Excitation (2.5-15):

Emission (2.5-20):

Scan Limits (nm)

Lower Limit (Ex):

Upper Limit (Ex):

Offset [Em-Ex]:

Data Interval:

The slit positions remain constant during the entirety of a synchronous scan.

Lower Limit	Range: 200 - 800 nm
Upper Limit	Range: 200 - 800 nm

The lower and upper limits determine across which band of wavelengths the monochromators are driven and signal intensities read. The limits are determined by the boundaries of the excitation monochromator (200-800 nm). If you request a scan from a lower limit of 450 nm to an upper limit of 650 nm with an offset of 15nm then the monochromators move as follows:

Excitation Monochromator	450 - 650 nm
Emission Monochromator	465 - 665 nm

The lower limit must be lower than the upper limit and the upper limit plus the offset must be less than the upper limit of emission monochromator.

Offset [Em-Ex]	Range: 0.1 to Upper Limit-Lower Limit
-----------------------	---------------------------------------

This is the offset (nm) between the excitation and emission monochromators. Synchronous scans are performed using constant wavelength separation. This means that as the monochromators move across the wavelength range the separation between them remains constant.

Data Interval	Range: 0.1 - 5.0 nm
----------------------	---------------------

Determines the data output interval for synchronous scans. As the monochromators scan across the range determined by the lower and upper limits data is collected at discrete intervals. The parameter determines how frequently a data point is collected and how many points are in a scan.

Example:

Lower Limit: 450 nm
Upper Limit: 650 nm
Data Interval: 0.5 nm

First data point: 450 nm
Second data point: 450.5 nm
⋮
Last data point: 650 nm

Total samples collected: $(650-450) / 0.5 + 1 = 401$ data points

Output File Format

DOS/Windows All records end with a CR/LF.

Macintosh All records end with a CR only.

Print Results Check this box if you desire a printout of the data as collected. The data does not print after each sample, but rather after a page full of data has been collected. The last page will be printed when the program is exited.

File Format
<input checked="" type="radio"/> DOS/Windows
<input type="radio"/> Macintosh
<input type="checkbox"/> Print Results

Delivery Method

Cuvette Samples are supplied for analysis via cuvette.

Autosampler Samples are supplied for analysis via the autosampler system. Press *AS Setup* button to enter autosampler parameters.

AS Setup

This button appears if the Autosampler option is selected. Clicking the button moves you into the diluter station and autosampler parameter selection dialog box (figure 2.5). The system supported by WINFAC uses a Perkin Elmer DS-6 diluter station equipped with a CAVRO high precision syringe pump. The diluter station is in line with a Perkin Elmer AS-91 robotic sample probe and Hellma Cells, Inc. high sensitivity flow cell. For a description of the autosampler system and the validation work on it refer to Schimmel et. al., 2000 (schimmel@u.washington.edu).

WINFAC

File System Checks

DILUTER SETTINGS

Sample Volume (ul): 750

Air Volume (ul): 500

Backfill Volume (ul): 0

Flush Vol (ul): 8000

Air Dry Time (secs): 15

Sample

Fill Speed : 15

Dispense Speed: 10

Flush

Fill Speed: 10

Dispense Speed: 10

Syringe Volume Resolution

☐ 5 ml ☒ Standard

☒ 10 ml ☐ Fine

AUTOSAMPLER SETTINGS

☒ Standard ☒ Blank

Pos. Freq.

1 50

2 50

Starting Tube: 5

Ending Tube: 27

Sample Disposition

☒ Throw Away

☐ Save

SAMPLING ARM POSITION

XY

Increment:

☐ 1 mm

☒ 10 mm

☐ Rinse

☐ Standard

☐ Start Tube

☐ End Tube

☒ Other 213

X: 0 Y: 21

Move

PROBE HEIGHT SETTINGS

Locate

Lower Probe

☐ Rinse

☐ Standard

☐ Stand. Air Fill

☐ Samples

☐ Sample Air Fill

Done

Figure 2.5 Diluter station and autosampler parameter dialog box.

Diluter Settings

Sample Volume Volume (μ l) of sample to extract from the sample vials.

Air Volume Volume (μ l) of air to push the sample back to the flow cell.

Backfill Volume Volume (μ l) in sample line used to push sample back toward the probe. If your system gets air bubbles in the flow cell you can take a larger sample volume, flush it slightly past the flow cell and then push the air bubble out of the flow cell by pushing the *Backfill Volume* amount of air out of the sample line.

Flush Volume Volume (μ l) of the solution that the system uses to flush the system. If a larger volume is requested than fits into the syringe, the syringe keeps filling and flushing until the requested amount is delivered through the system.

Air Dry Time Time (seconds) that the system is air dried after being flushed at the completion of each sample.

Sample Settings

Fill speed Speed that the syringe draws sample out of the tubes and air into the system to move the sample back to the flow cell. Larger numbers slow down the syringe while smaller numbers speed it up. Drawing sample in too quickly may introduce air bubbles to the system, whereas drawing too slowly needlessly adds time to each sample analysis. Range: 1-40.

Dispense Speed Same as Fill Speed except this is the speed that sample solution is pushed out of the system. Range: 1-40.

Flush Settings

Fill speed Same as Sample Settings Fill Speed for samples except this is the speed with which the syringe draws in the flush solution. Range: 1-40.

Dispense Speed Same as Sample Settings Fill Speed except this is speed that flush solution is pushed out of the system. Range: 1-40.

The screenshot shows a window titled "DILUTER SETTINGS" with the following fields and values:

Parameter	Value
Sample Volume (μ l):	750
Air Volume (μ l):	500
Backfill Volume (μ l):	0
Flush Vol (μ l):	8000
Air Dry Time (secs):	15
Sample	
Fill Speed :	15
Dispense Speed:	10
Flush	
Fill Speed:	10
Dispense Speed:	10

Syringe Volume

The Cavro syringe pump is able to use either 5 ml or 10 ml syringes. This option selects which size syringe is on board.

Resolution

The Cavro syringe pump is capable of extremely fine stepping. This setting tells the program which resolution you wish to use. For the purposes of delivering sample to the flow cell, the Standard setting is more than adequate.

Autosampler Settings

Standard

If Standard solution (Control) is available indicate this by selecting (checking) the appropriate checkbox. If available and to be used (checked) the following two variables must be filled in.

	Pos.	Freq.
<input checked="" type="checkbox"/> Standard	1	50
<input checked="" type="checkbox"/> Blank	2	50
Starting Tube:		5
Ending Tube:		27
Sample Disposition		
<input checked="" type="radio"/> Throw Away		
<input type="radio"/> Save		

Pos Tray position of the Standard stock solution.

Freq Frequency to sample the stock solution. If Standard solution is present the program automatically samples the stock solution at the start and end of each tray. The frequency indicator declares how often during the running of the tray to resample the stock solution. In example above 50 indicates to resample the stock solution after every 50 analysis samples.

Blank (Solvent Blank)

If solvent blank solution (solvent only) is available indicate this by checking the box. If available and to be used the following two variables must be filled in.

Pos Tray position of the solvent blank stock solution.

Freq Frequency to sample the stock solution. If solvent blank solution is present the program automatically samples the stock solution at the beginning and end of each tray. The frequency indicator declares how often during the running of the tray to resample the stock solution. In example above 50 indicates to resample the stock solution after every 50 analysis samples.

Starting Tube: Tube position of the first non-Standard and non-solvent blank sample to be analyzed. If this is the first tray in the experiment and pure samples are required the first pure sample must be in this position.

Ending Tube: Tube position of the last sample to be analyzed.

Sample Disposition

Declare how the sample should be handled after the fluorescent intensities have been determined.

Throw Away Move armature to the Rinse position before pushing out the sample.

Save Leave armature in the sample tube and push out the sample. The amount of sample recovered is less than the amount extracted as some remains trapped in the flow cell until the system flush. Standard and Blank samples are never put back into the stock solution regardless of this setting. They are always thrown away to maintain the purity of those samples.

Sampling Arm Positioning

This section allows the user to fine-tune the X-Y coordinates of the tube positions. **Note:** The X-Y coordinates determined using this capability are **not saved**. You can not go into this section and save the optimal positions for the various tube positions. This section is useful for initial determination of the geometry of a tray full of tubes. The tray geometry file however must still be created using any text editor. See Appendix A for a description of the tray geometry file structure.

SAMPLING ARM POSITION

XY

Increment:

- ☐ 1 mm
- ☒ 10 mm

☐ Rinse

☐ Standard

☐ Start Tube

☐ End Tube

☒ Other

X: Y:

Move

AS-91 autosampler XY ranges:

X: 0 - 302 mm
Y: 0 - 181 mm

The navigation buttons are not displayed unless you click on one of the location radio buttons. The home position of the armature is shown on the diagram. Each of the navigation buttons moves the probe in the direction shown by the arrows relative to that orientation of the autosampler.

Increment Declares the incremental unit for moving the armature and/or probe arm (1 or 10 mm) when clicking one of the navigation buttons.

Rinse Probe is located at the rinse position, directly over the small washing cylinder located in the disposal cup.

Standard Probe is located over the tube location listed in the Standard Position box (see above).

<u>Start Tube</u>	First tube position to be sampled that is not a Standard or Blank.
<u>End Tube</u>	Last sample tube position to be sampled.
<u>Other</u>	Number of the tube to move to prior to clicking the radio button. Once the radio button has been selected you may move to other positions by typing in the number of the tube to locate and then press the Move button. In addition to moving the probe the Move button also causes the probe to lower to the Sample Air Fill height (see Probe Height Settings below).

Probe Height Settings

These settings determine how the probe operates during the sampling procedure. The edit boxes are available only if you have clicked on one of the radio buttons in this section. All numbers are in mm with larger numbers lowering the probe further than smaller numbers. The range is between 0 (probe at highest position) and 146 (probe at lowest position).

PROBE HEIGHT SETTINGS

Locate	70	<input type="radio"/> Rinse
	100	<input type="radio"/> Standard
	30	<input type="radio"/> Stand. Air Fill
Lower Probe	105	<input checked="" type="radio"/> Samples
	70	<input type="radio"/> Sample Air Fill

Clicking on any of the radio buttons moves the probe to that location, but does not automatically lower the probe to the assigned height listed beside the radio button. Press the *Lower Probe* button to lower the probe to the height listed beside the radio button selected.

<u>Rinse</u>	Probe is located at the rinse position, directly over the small washing cylinder located in the disposal cup.
<u>Standard</u>	Probe is located over the tube location listed in the Standard Position box (see above). Height at which the probe is lowered for sampling both standards and solvent blanks.
<u>Standard Air Fill</u>	Same as Standard. Height of the probe prior to pushing sample back to the flow cell through introduction of room air.
<u>Samples</u>	Probe is located at the position of the starting tube. Height of the probe when lowered for sampling.
<u>Sample Air Fill</u>	Same as Samples. Height of the probe prior to pushing sample back to the flow cell through introduction of room air.

Lower Probe

This button lowers the probe to the height shown in the Sample Air Fill edit box at the current location.

Locate

This button moves the autosampler to each tube location between the Starting and Ending Tube positions shown in the Autosampler Settings sections. The probe moves to a location, lowers to the height declared in the Sample Air Fill edit box and then raises up and moves to the next location. This is helpful when verifying that all the XY coordinates in the tray geometry file are accurate before starting analysis runs.

Menu Options

Tray Geometry File (.tgf)

The autosampler requires that there be a Tray Geometry File to define the XY locations of all tube locations on the tray. If you change to a tray system with a different geometry use this option to select the desired geometry file. See Appendix A for the structure of the tray geometry file. Tray geometry files are saved in the METHODS subdirectory.



Check Rinse

This option runs through a normal rinse and air dry cycle to verify that the amount of flush solution and air dry time are adequate.



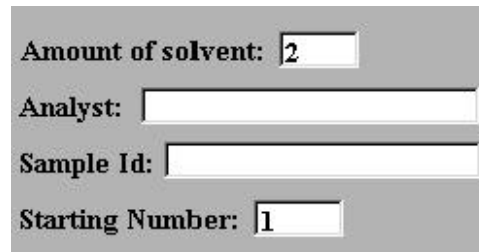
Check Volumes

This option performs a sample draw from the Starting Tube location and stops once the sample has been drawn back to the flow cell. You are then prompted to check the flow cell and verify that it is full and without bubbles.



Identification Section

These parameters are present to save identifying information in the data file. Information **must** be present in all edit boxes before you can proceed.



The screenshot shows a form with four input fields. The first field is labeled 'Amount of solvent:' and contains the number '2'. The second field is labeled 'Analyst:' and is empty. The third field is labeled 'Sample Id:' and is empty. The fourth field is labeled 'Starting Number:' and contains the number '1'.

Amount of solvent The amount of solvent used in each sample in ml. This is critical to know in that it must match the amount of solvent used when the pure samples were created if you want FMSPILL to output accurate estimations of the number of microspheres present in a sample. If dissolving 250 microspheres in 2 ml of solvent created a pure sample, but 1 ml is indicated on the form, results output from the FMSPILL program will double the estimate of microspheres present in the sample. See FMSPILL manual for further discussion.

Analyst The person responsible for performing the analysis.

Sample ID Any identifying information desired.

Starting Number

Number of the first non-pure sample to be read. In most cases this will be 1, which is the default. It is important to note that when you append to the data file the next sequential number after the last record in the file is shown in this box. If for some reason you need to start the sample number at a different number than the one presented when you select the **Append Data File** menu option, **do not** edit the method file and change the starting number. As soon as a method is edited the program assumes a new analysis is starting and requests that a new method and data file be saved. If the data file is saved under the same name the program warns that the existing data file will be overwritten and **all data already saved lost**. To change the starting number of a tray without overwriting existing files please use the **Reset Sample Number** sub-menu described in the Running Samples section.

Cancel

To cancel a method definition, press the **C**ancel button. The form is erased and a blank backdrop once again presented.

Save As

When the form is complete press the **S**ave As button to indicate you are finished. At that point numerous checks are made to verify that the entries made are within established ranges. If any problems are detected a dialog box displays the problem and once acknowledged by the operator the invalid parameter is highlighted for correction. If all checks pass you are prompted with a Save As dialog box to enter the name of your method (fig 2-6).

Method files have a .MTH extension that is automatically appended to the filename. For example if you wish to name a method file as TESTCUV.MTH you need only type in the TESTCUV into the File Name edit box.

If you are saving a method file as a name that already exists a dialog box is presented offering you the choice of replacing the saved file (Yes) or not replacing the saved file (No) (fig 2-7).

You are then prompted to save the output data file (fig 2-8). All .FIX files are listed unless you are running only a synchronous scan analysis. In that case all .SYN files are listed. As in saving the method file just type in the filename and the program will append the correct extension(s). WINFAC will create up to six files depending on which Scan Type(s) you have selected. For example if you enter a base filename of TESTCUV the following files will be created:

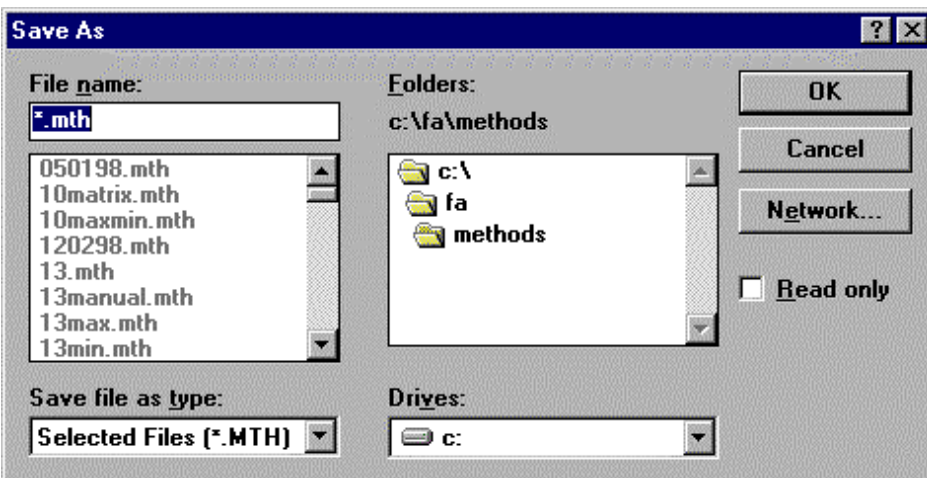


Figure 2-6. Save As dialog box for a methods file.

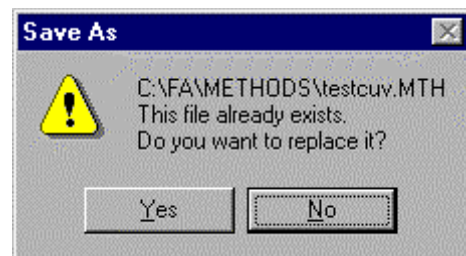


Figure 2-7. File exists dialog box

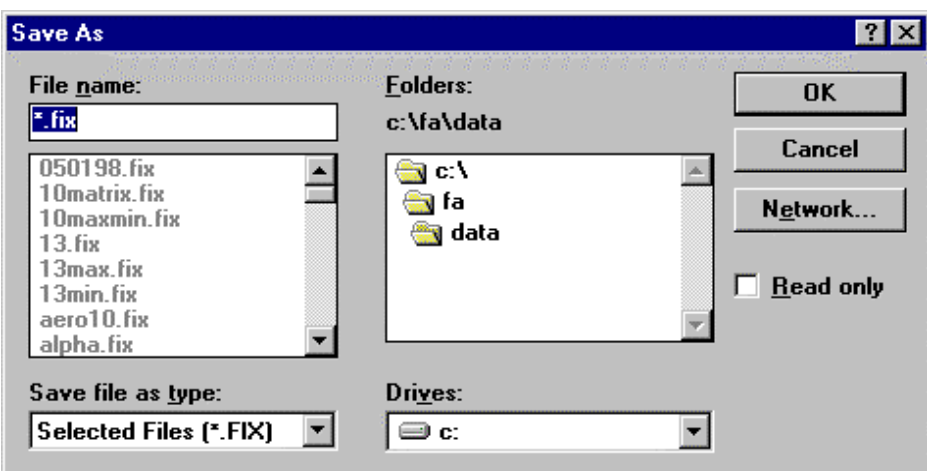


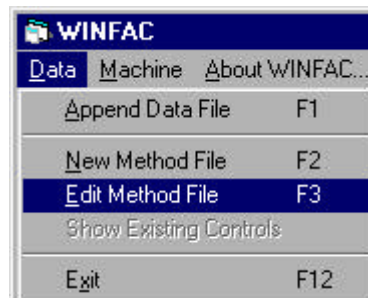
Figure 2-8 Save As dialog box for a data file.

<u>Filename</u>	<u>Scan Type</u>	<u>Contents</u>
TESTCUV.FIX	FIXED, BOTH	Sample Data
TESTCUV.CTL	FIXED, BOTH	Control Readings
TESTCUV.BLK	FIXED, BOTH	Blank Solvent Readings
TESTCUV.SYN	SYNCHRONOUS, BOTH	Sample Data Spectrum
TESTCUV.STL	SYNCHRONOUS, BOTH	Control Reading Spectrum
TESTCUV.SLK	SYNCHRONOUS, BOTH	Blank Solvent Spectrum

As with method files, if you are saving a data file group with a name that already exists, a dialog box is presented offering you the choice of replacing the saved file (group) (**Yes**) or not replacing the saved file (group) (**No**) (fig 2-7). It is important to recognize that responding with **Yes** completely and **irretrievably erases** any data files with that name.

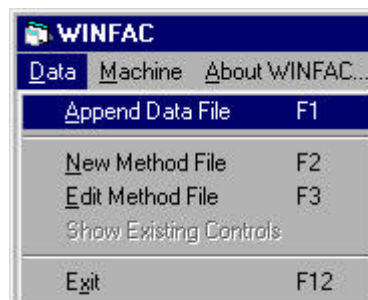
Editing A Method File

To edit a method, select the **Edit Method File** sub-menu from the **Data** menu. See the description of parameters under *Create A Method File* for explanations of parameters required for the form. Take note that if you change a synchronous scan method to a fixed wavelength method you will be required to fill in wavelength information. Since you have selected the **Edit Method File** option you will be prompted for a new method filename when you hit the Save As button. You can overwrite the existing method or enter a new method name. You are immediately prompted for a new data filename. Please beware that should you replace an existing data file, the former file is **irretrievably lost**.



Append Data File

If your analysis is large enough that it is impossible to complete during one session in front of the spectrofluorimeter WINFAC offers the ability to continue an analysis at a future time. We highly recommend that if you continue an analysis at a different time that you immediately run blank solvent samples and control samples to assure that spectrofluorimeter is functioning as before. If the autosampler is used, the program automatically runs these checks if solvent blanks and control samples are present on the tray. To continue existing analyses select the **Append Data File** sub-menu from the **Data** menu. The program displays a file selection dialog box with all existing .syn and .fix files showing. Select the file you wish to append to and the program opens to a display of the existing method file. If you are performing an analysis that uses both fixed wavelength and synchronous scans you may open either the .syn or .fix file to reinitiate the analysis. The program reads the attached method file to ascertain which scans are needed. All options on the displayed method are disabled (grayed out) to prevent changing a method in mid analysis. The starting number display should reflect the number of the last sample in the file plus one. Press the **OK** button to continue.



Check Existing Controls

Once you have selected Append File and loaded an existing dataset you may check the status of existing Controls and/or Blanks for that dataset by selecting **Show Existing Controls**. See Figure 3-4 and accompanying description for an explanation of the trend display screen.



Running Samples

Overview

Once the method and data files are created and saved, the process of running samples is ready to begin. First however, you need to know the processes involved in analyzing a sample as well as a few tips & tricks when analyzing samples.

Spectrofluorimeter Warm-Up

It is highly recommended that the spectrofluorimeter be allowed to warm-up for 7-10 minutes after the **[I]nit** button is pressed (see below). This allows the electronics to stabilize in the machine and improves the stability and reliability of results.

Sample Analysis Process

It is helpful to have a minimal understanding of the process involved in analyzing a sample once the computer has been told to read a sample. Following is an overview of the steps needed to process a sample. The method of delivery to the system is irrelevant (cuvette (manual) or flow cell (automated)) as the process to analyze the sample is identical. Once the sample is in place and the **[R]ead** button pressed the following happens:

If fixed wavelength intensity reading(s) are requested the following steps are performed until all requested fluorescent dyes are process:

1. Verify excitation and emission slit positions and reset if necessary.
2. Move monochromators to the next requested wavelength pair setting.
3. Send a request to the spectrofluorimeter to retrieve an intensity reading based on the current parameters.
4. Increment fluorescent dye counter and return to step 1 if more dyes remain to be read, otherwise prompt user for action.

If a synchronous scan is requested:

1. Verify excitation and emission slit positions and reset if necessary. This will only happen if you are using both fixed wavelength and synchronous scan analysis since you can not have multiple slit settings for synchronous scans.
2. Set excitation monochromator lower and upper limits. Set emission monochromator lower and upper limits.
3. Move monochromators to starting positions.
4. Retrieve a synchronous scan status record to start the scan.
5. Scan across the defined range of wavelengths and display the spectrum on screen.
6. Prompt user for action.

Key Equivalents

Many of the buttons shown in the analysis section of the program have key equivalents to reduce the need for a mouse. Any underlined character within a button is accessible by holding down the **Alt** key while pressing the underlined character key. Many of these same keys may also be pressed without holding down the **Alt** key. Messages are relayed to you within the yellow progress box. If a character is surrounded by brackets [] it may be pressed without the **Alt** key. For example in figure 3-1 the **[I]nit** button may be “clicked” by pressing the **[I]** key or the **[Q]uit** button may be clicked by pressing the **[Q]** button. Key presses may be in either upper or lower case.

Standards and Blanks

As with any electronic device there may be drift in the electronics of the spectrofluorimeter over the duration of an analysis run and certainly between runs performed temporally disconnected from each other. Since it is a rare time when all samples from a single experiment can be analyzed during one sitting at the spectrofluorimeter we introduced running both standards (controls) and blanks (solvent blanks) periodically through the analysis to verify the stability of the spectrofluorimeter. Please see

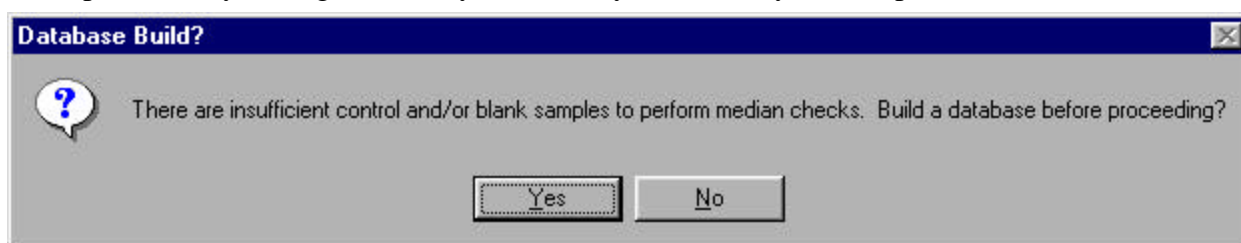


Figure 3-1. Build the Control and Blank database query request.

Section 3 of the *Manual for Using Fluorescent Microspheres to Measure Regional Organ Perfusion* available from the FMRC web site for an in depth discussion of standards. However, at the beginning of an experiment there is no information to use as a check against the current Control and/or Blank sample, and the program queries whether to build up a database before proceeding (fig 3-1). If you respond with **[Y]es** the autosampler repeatedly samples the Standard and Blank samples alternately until the database is sufficient (currently the minimum records required is five for each of the validation solutions). If using cuvettes only the **[B]lank** and **[C]ontrol** responses are activated until the minimum number of Standards and Blanks are read.

Subsequently, when Controls are read using fixed wavelength analysis

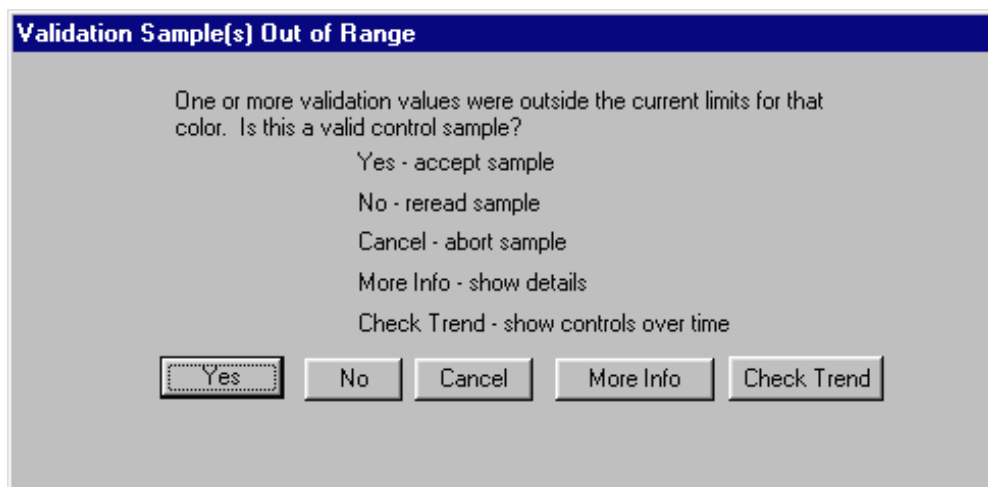


Figure 3-2. Validation sample of range notification dialog box.

the results are compared against the database of information available for each dye setting. The comparison is made against the current standard deviation of previous control samples. If the current reading is outside three standard deviations from the mean, the sample is highlighted in green, but the sample is not aborted. Once the value for a wavelength pair exceeds four standard deviations from the mean, the offending values are highlighted in red and a dialog box appears indicating that there is a problem (fig 3-2). At this point human intervention is required to continue processing. Options are:

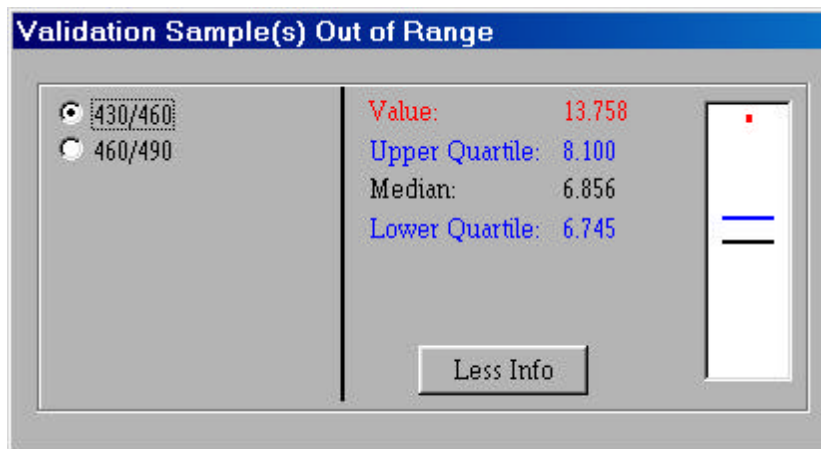


Figure 3-3. Expanded view of validation sample out of range. Graphic on right shows the median, upper and lower quartiles and the value of the sample out of range.

Yes: Accept the sample as a valid control.

No: Reread the sample. Useful if trying to read a control, but inadvertently read a different sample.

Cancel: Abort this sample. If using the autosampler it ignores the Control request and proceeds to next Blank or analysis sample that is scheduled.

More Info: If pressed a dialog box appears (fig 3-3) which gives expanded information on each of the dyes that are out of range. This is in box plot format with the median indicated in black, the two interquartile distances indicated in blue, and the offending value displayed in red.

Check Trend: Displays all controls/blanks for the current analysis. This may visually give the user a better grasp of what is happening during operation of the spectrofluorimeter (fig 3-4). When Check Trend is invoked from an error situation the Control value causing the problem is not displayed graphically, but is rather written out in text just below the **OK** button. This avoids the problem of implying the Control has been accepted by displaying it graphically.

The sampling screen provides for a median line at the top of the display (fig 3-5). Once a sufficient database is established this line displays the current median value of all control samples read thus far for this experiment. It provides for a quick visual reference for the operator whenever control samples are read to ascertain the current state of the spectrofluorimeter.

The number of the current sample is used for the records written into the control and blank files to enable the user to identify when the control or blank was read during the analysis. The sample

number is not increased during a Standard or Blank read in order to keep the samples numbered sequentially. In the example presented (fig 3-5) the NS indicates "No Sample" as that sample was rejected because it was out of range.

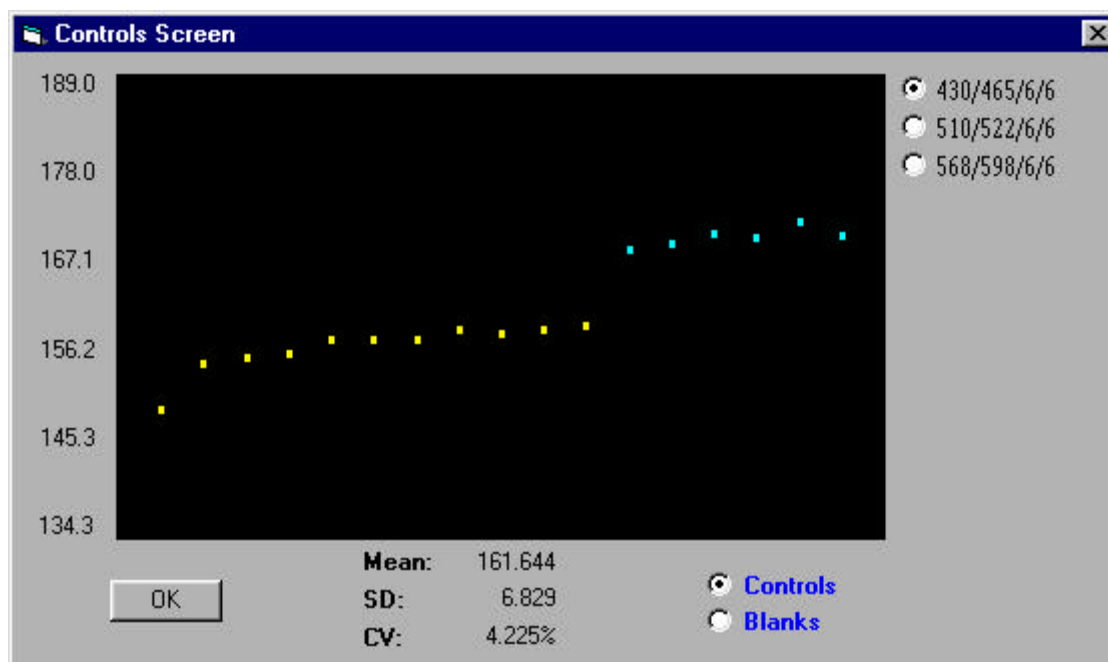


Figure: 3-4. Trend display screen. Controls/blanks from different sessions/days are displayed in different colors to facilitate analysis of possible electronic drift/standard problems.

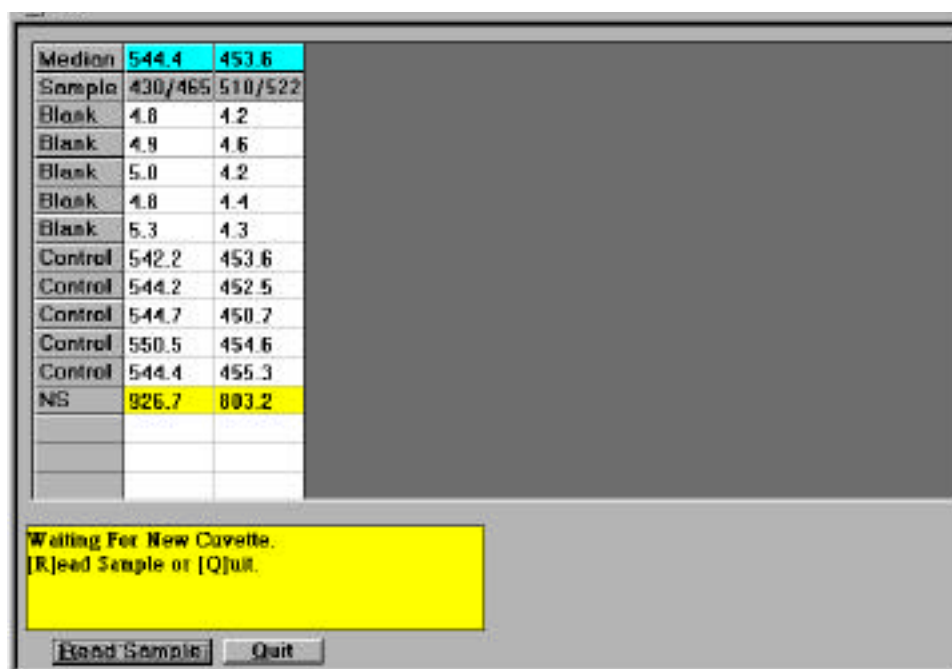


Figure 3-5. Sampling screen showing results of reading blanks and control samples.

Pure Samples

When starting a new experiment the program checks whether the **Generate a Spillover** option was selected in the method definition. If selected the operator is queried whether to read pure samples prior to analyzing the experiment samples (fig 3-6). The Cancel option is present to handle those instances when it is easier to use an existing method with a spillover generation request, but in fact pure samples are not required.

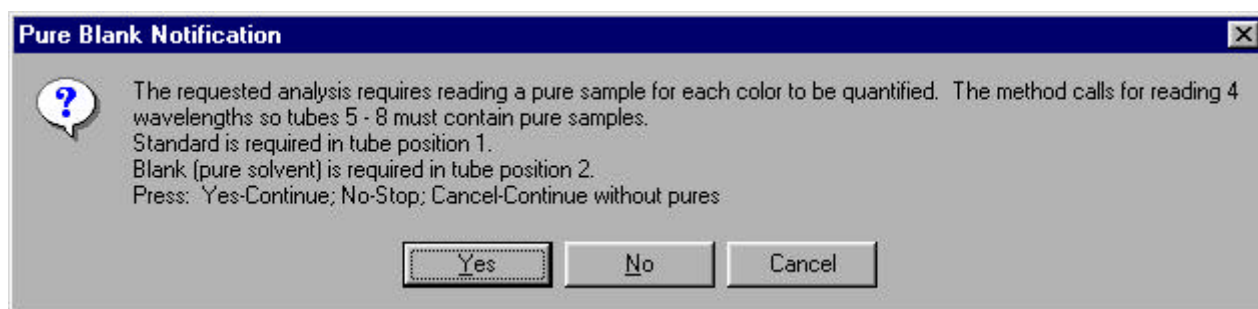


Figure 3-6. Pure and blank needed notification box.

System Initialization Commands

The following commands are sent to the spectrofluorimeter whenever the method file is loaded or reloaded.

Free Run Mode	Spectrofluorimeter runs without prompts.
Fluorescence Mode	Sets LS-50 (B) to fluorescence mode.
Turn Lamp On	Causes the source to be turned on.
Emission Filter	The filter wheel is moved to the specified position.
PMT Voltage	Set the PMT voltage to be used . If the default voltage is used the PMT voltage is defined according to the excitation slit position used for each dye setting.
Scan Speed	Set speed for scans.

Initializing the System

Once the method and data files are saved the program hides the method declaration screen and displays the sampling screen (fig 3-6). The spectrofluorimeter must be initialized with those parameters that are constant throughout an analytic cycle prior to proceeding. This is accomplished by pressing the **[I]nit** button or pressing the **I** key as shown in figure 3-7. Details of what the program is doing during the initialization process are explained in the preceding section. When initialization is complete the system is ready for use (fig. 3-8).

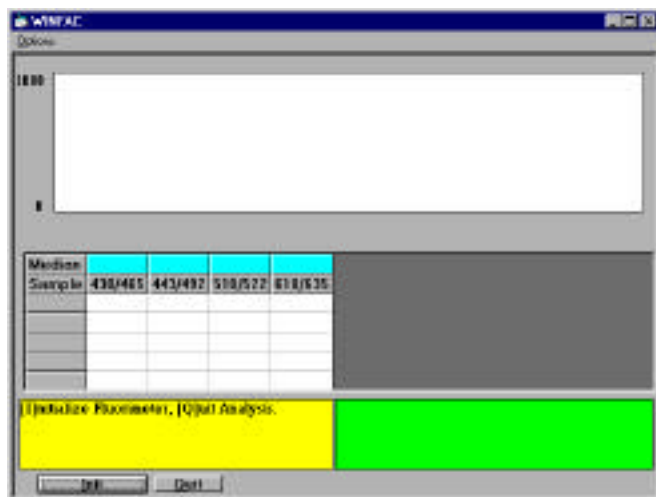


Figure 3-7. Initial screen showing both a fixed wavelength and synchronous scan screen request for four fluorescent dyes.

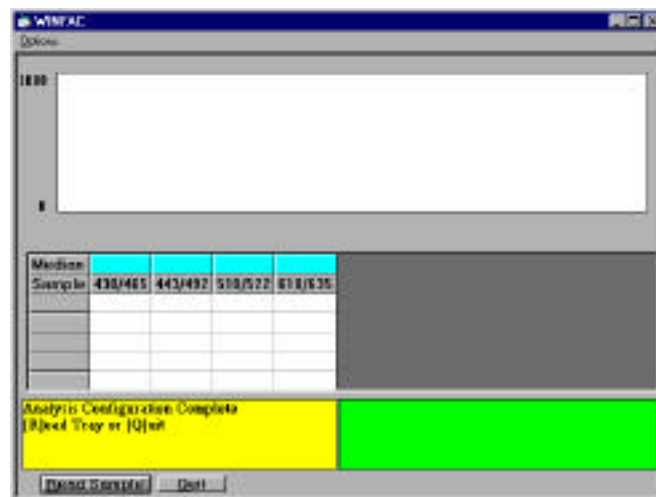


Figure 3-8. Screen after initialization is completed and awaiting start of analysis. Prompt differs slightly if using a cuvette for sample delivery.

The description presented here is for using a cuvette to deliver the sample to the system. If using the autosampler system once you press the **[R]ead Tray** button the system functions automatically and no further intervention other than occasional verification of continued operation should be necessary.

To start the analysis insert a cuvette in the cuvette reader, close the reader door, and press the **[R]** key or click the **[R]ead Sample** button. Progress messages are displayed in the yellow progress box and when the sample analysis is finished you are invited to enter Comments in the comment edit box. You must also indicate how to handle the sample that was just read (fig 3-9). The four available options are:

- [A]ccept:** the sample intensity readings are written to the data file.
- [R]eject:** program prompts with options to reread the sample or cancel the reading of that sample.
- [C]ontrol:** sample intensity readings are written to the controls file.
- [B]lank:** sample intensity readings are written to the blanks file.

The screenshot shows the WINFAC Options dialog box. It contains a table with sample data and a prompt for comments.

Median	544.4	453.6
Sample	430/465	510/522
Blank	4.8	4.2
Blank	4.9	4.6
Blank	5.0	4.2
Blank	4.8	4.4
Blank	5.3	4.3
Control	542.2	453.6
Control	544.2	452.5
Control	544.7	450.7
Control	550.5	454.6
Control	544.4	455.3
NS	926.7	803.2
1	920.4	798.8

Enter Comments NOW if needed
[A]ccept/[R]eject/[C]ontrol/[B]lank

Buttons: **Accept** **Reject** **Control** **Blank** **Comment** []

Figure 3-9. Sampling screen prompting operator for type of sample. Example is for fixed wavelength analysis.

Options A, C and B are each followed with either an invitation to present a new sample for analysis or if you are also running synchronous scans the synchronous scan is performed and you are given an option to **[A]ccept** or **[R]eject** that scan. If you reject after the synchronous scan the fixed wavelength analysis is also rejected.

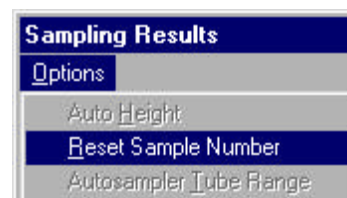
Autosampler Options

WINFAC has two options which are useful during autosampler operations. Should you need to abort a run press the **F5** key and the autosampler will stop at the end of the current sample being analyzed and query whether you really want to abort. Should you need to pause the operation press the **F11** key and the autosampler will stop at the end of the current sample being analyzed. Press **F11** again to resume processing.

Sampling Options

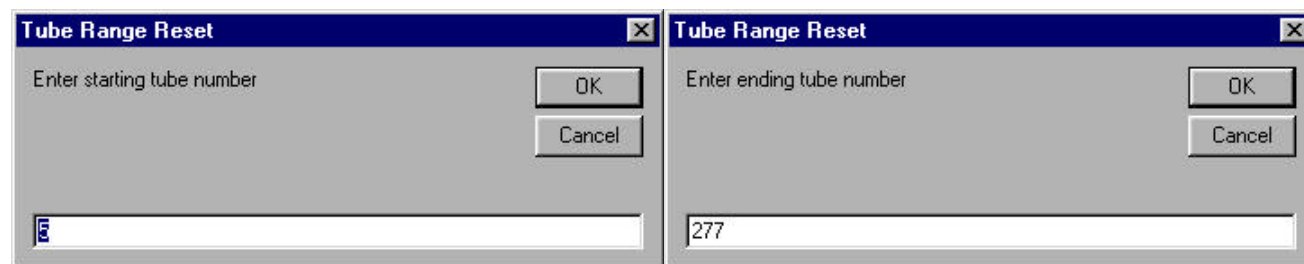
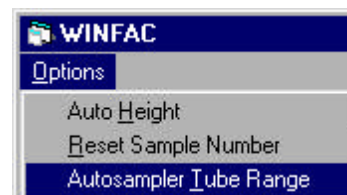
Reset Sample Number

There may be instances when the default number of the next sample is not what you wish it to be. An example of such an instance is if you need to reread some samples after diluting them. In this instance select the **Reset Sample Number** sub-menu under the **Options** menu prior to reading the sample. You are prompted to input the new sample number. It is important to remember that if you reread a sample at a later date the former reading **is NOT removed**. All readings are made sequentially and the output data file is a sequential listing of the results. You will need to edit the data file through a text editor or spreadsheet in order to remove the earlier reading.



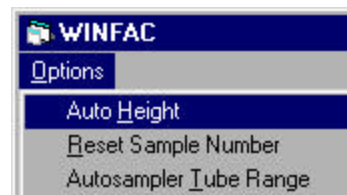
Autosampler Tube Range

When a method file is defined which utilizes the autosampler the Starting and Ending tube numbers of the initial tray are stored (See **AS Setup** above). If you modify the method file to change the Starting and Ending Tube numbers the program prompts you to overwrite the existing method and data file which will irretrievably lose all data previously acquired. To avoid this, use the **Autosampler Tube Range** sub-menu under the **Options** menu prior to starting to read subsequent trays. Two dialog boxes (shown below) are displayed asking for the starting and ending tube numbers. Enter the appropriate numbers for the current tray.



Auto Height

When performing synchronous scans it is sometimes useful to expand a spectrum to the full height of the scan window. The window defaults to the full range of the spectrometer (0-1000), however, if you have a weak signal and wish to amplify it select the **Auto Height** sub-menu under the **Options** menu after the synchronous scan is complete. The Y axis is modified to better display the scan just performed (fig 3-10).



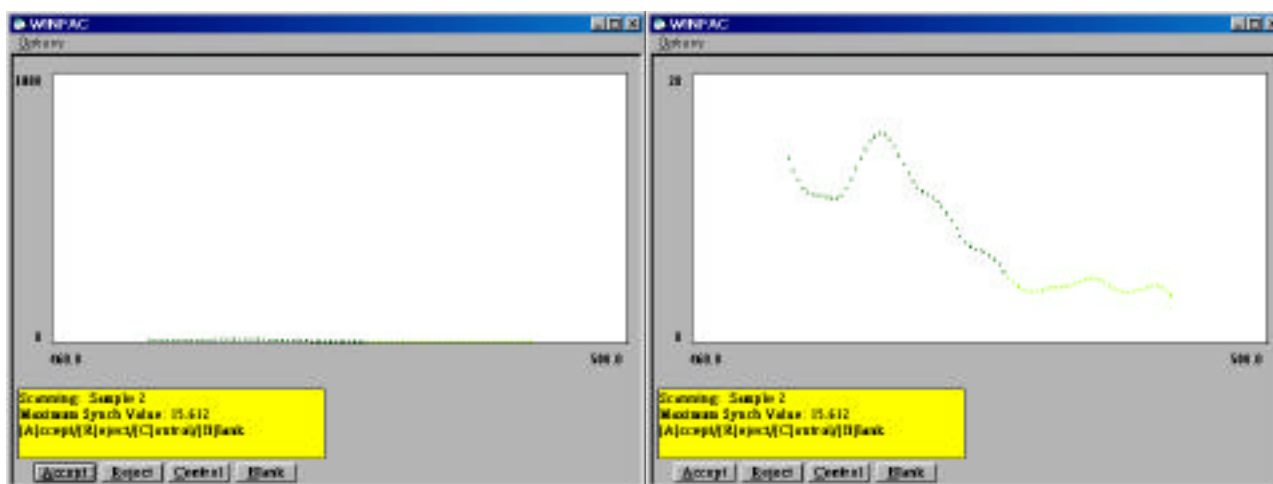


Figure 3-10 Synchronous scan before and after application of the Auto Height option.

Machine Options

Overview

The options described below are available when the program initially boots up or at any time when there is a blank backdrop (no method description, autosampler setup, or sampling screen).

Run Watercell

It is recommended that you verify the status of the spectrofluorimeter whenever you start a day of analysis. One of the best methods to accomplish this is by running a time drive with a watercell inserted in the cuvette holder. You can run a water cell time drive by selecting the **Run Watercell** sub-menu from the **Machine** menu. The initial screen prompts you to insert the watercell (fig 4-1). The program



uses a predefined set of parameters (table 4-1) and averages the values over a two minute period. The results of previous watercell runs are saved in a file named WINFAC.MSF stored in the main directory as listed in section 1.4. If there are five or more watercell time drive reports in the file those data are averaged and compared against the results of the current time drive. Prior to the fifth run all values are accepted as the database of acceptable results has not yet been established. If the current time drive results compare favorably with previous operations the program will report something along the order of figure 4-2. If, for some reason, the spectrofluorimeter gives readings indicating a change in status of the spectrofluorimeter a report along the lines of figure 4-3 indicates how far from the expected the readings lie. Currently time drive

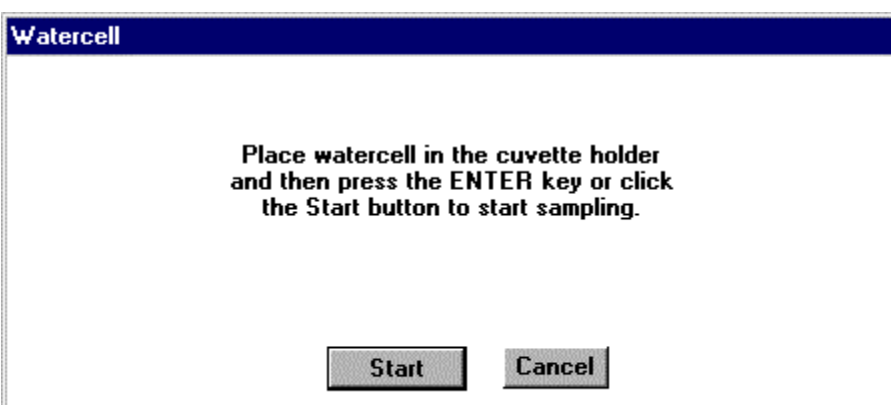


Figure 4-1. Initial watercell screen.

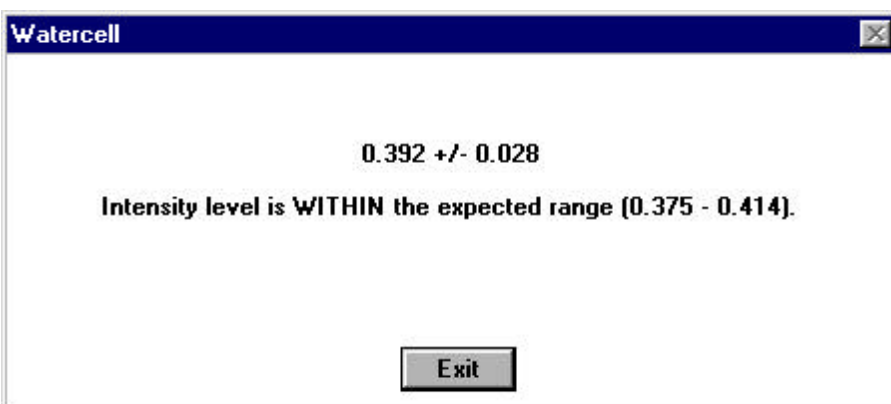


Figure 4.2 Screen indicating a time drive where the results are within the expected range for the spectrofluorimeter.

Currently time drive

intensities that differ more than 10% from the mean of previous time drives are flagged. Our experience indicates that if the spectrofluorimeter is not functioning properly it is much more than 10% away from the mean. If out of bounds please verify that the watercell is truly inserted in the cuvette reader. If not, place the watercell in

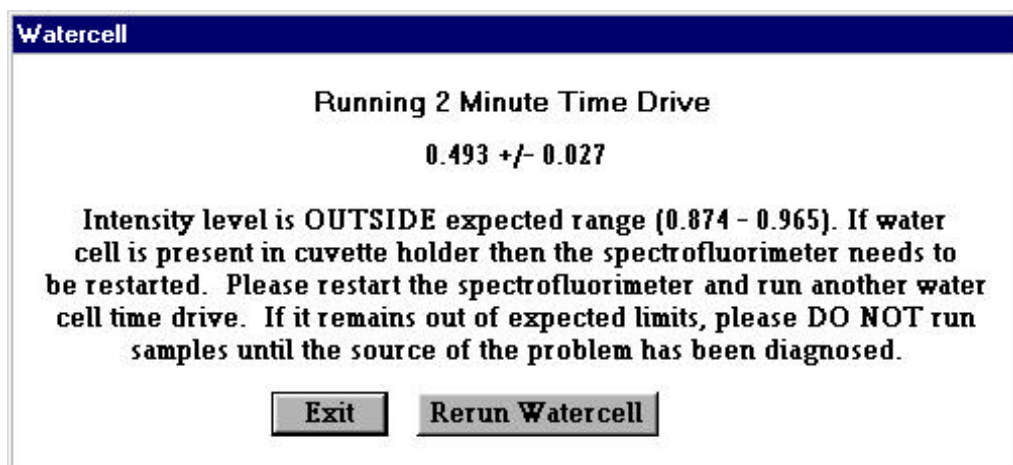


Figure 4.3 Screen showing the results of a time drive indicating that something is possibly wrong with the spectrofluorimeter operations.

the reader and press the **Rerun Watercell** button. Otherwise try turning the spectrofluorimeter off, wait 20-30 seconds, turn it back on and then try the **Run Watercell** option again. This generally cures the problem. If not there may be something wrong with the spectrofluorimeter which should be addressed prior to using the machine for analysis.

If the results are within the 10% error margin the variables in table 4-2 are stored in WINFAC.MSF:

Intensity Check

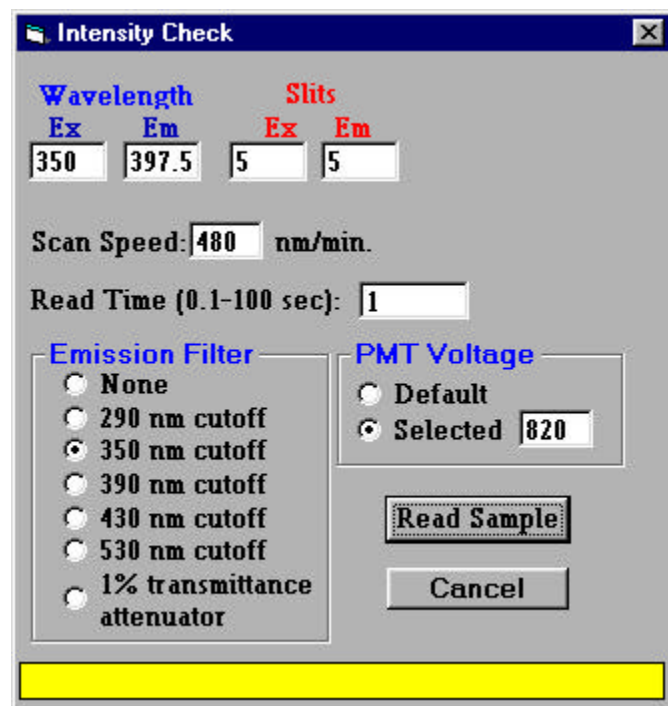


Figure 4-4. Intensity Check dialog box.



This option allows you to check the intensity levels of a sample without having to set up an entire method file. Select the **Intensity Check** sub-menu from the **Machine** menu. The dialog box prompts you for the parameters under which to run the intensity check (fig 4-4). See the description of parameters under **The Method File** for the specifics on how to fill out this form. Once the parameters are set click the **Read Sample** button to read one intensity level. The program displays the progress of the reading in the yellow results box. The intensity level is also displayed in this box at the completion of the operation. Press the **Exit** button to close the Intensity Check dialog box.

Machine Configuration

The WINFAC Setup form allows you to modify the hardware configuration of your fluorimetry system that was declared during program installation. You can bring up WINFAC Setup form by selecting the **Setup Spectrofluorimeter** sub-menu from the **Machine** menu.

Following are descriptions of the information required on the *Setup Form* (figure 4-5).

Figure 4-5. Hardware configuration form.

Spectrofluorimeter - Click the radio button in front of the model spectrofluorimeter that is on your system.

Spectrofluorimeter COM Port - Click the radio button in front of the COM port to which your spectrofluorimeter is cabled.

Printer Available - Check the box if there is a printer available on the computer system.

Base Directory - Lists the main directory used by the program. Inside this base directory are the METHODS and DATA subdirectories.

Autosampler Available - Check the box if there is an autosampler system available. If checked the Setup dialog box expands to display other required items (fig 4-6).

Diluter COM Port - Click the radio button in front of the COM port to which the diluter station is cabled.

Autosampler COM Port - Click the radio button in front of the COM port to which the AS-91 is cabled.

The image shows a Windows-style dialog box titled "WINFAC Setup". It contains several sections for configuring hardware. The "Fluorimeter" section has two radio buttons: "Perkin Elmer LS50" and "Perkin Elmer LS50B", with the latter selected. The "Fluorimeter COM Port" section has four radio buttons: "COM1", "COM3", "COM2", and "COM4", with "COM1" selected. The "Dilutor COM Port" section has four radio buttons: "COM1", "COM3", "COM2", and "COM4", with "COM2" selected. The "AutoSampler COM Port" section has four radio buttons: "COM1", "COM3", "COM2", and "COM4", with "COM3" selected. There are "OK" and "Cancel" buttons on the right. Below these, there is a checkbox for "Printer Available" (unchecked) and a checkbox for "Autosampler Available" (checked). To the right of the "Autosampler Available" checkbox is a text field labeled "Base Directory" containing the path "C:\Program Files\DevStudio\VB". At the bottom, there is a section labeled "Autosampler" with a "Choose File" button. Below that, a label "Plate Description" is followed by the file path "C:\Program Files\DevStudio\VB\METHODS\New277r.tgf".

WINFAC Setup

Fluorimeter

☐ Perkin Elmer LS50

☒ Perkin Elmer LS50B

Fluorimeter COM Port

☒ COM1 ☐ COM3

☐ COM2 ☐ COM4

Dilutor COM Port

☐ COM1 ☐ COM3

☒ COM2 ☐ COM4

AutoSampler COM Port

☐ COM1 ☒ COM3

☐ COM2 ☐ COM4

☐ Printer Available

☒ Autosampler Available

Base Directory

C:\Program Files\DevStudio\VB

Autosampler

Choose File

Plate Description C:\Program Files\DevStudio\VB\METHODS\New277r.tgf

OK

Cancel

Figure 4-6. Hardware configuration form if the autosampler is selected.

History, Support, References

HISTORY

- WINFAC Change to Windows 95/98 interface
- Add ability to configure different slit widths for each excitation-emission wavelength
- FAC8 Add support for configurable wellplate reader
- Add automatic checking for controls out of range
- FAC7E Add support for controls.
- FAC7B Add read time and scan speed support

SUPPORT

In an attempt to limit the number of different versions of this program and to coordinate its evolution for all users, the Fluorescent Microsphere Resource Center will support WINFAC.

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The FMRC is keenly interested in making WINFAC useful to researchers using fluorescent microspheres. Any suggestions to enhance the program or this documentation will be gladly considered.

REFERENCES

Schimmel, C, D Frazer, SR Huckins and RW Glenny. Validation of automated spectrofluorimetry for measurement of regional organ perfusion using fluorescent microspheres. Comput Methods Programs Biomed, 62(2):115-25, 2000.

Appendix A. Tray Geometry File Structure

There are three numbers per tube position separated by commas.

X, Y, Tube#

X is the distance in the long direction of the autosampler (entire armature must move) whereas Y describes the shorter length (armature stationary, but the probe moves out on the arm). The first record in the file must be the location of the rinse cup and must be numbered as tube 0.

```
0,19,0
0,67,1
0,105,2
0,143,3
0,181,4
22,0,5
37,0,6
52,0,7
67,0,8
82,0,9
97,0,10
113,0,11
128,0,12
143,0,13
159,0,14
174,0,15
189,0,16
205,0,17
220,0,18
235,0,19
251,0,20
266,0,21
281,0,22
296,0,23
29,13,24
45,13,25
60,13,26
75,13,27
90,13,28
106,13,29
121,13,30
136,13,31
⋮
```