FFFHydRad 2.0

User's Manual

What is FFFHydRad?

Flow Field-Flow Fractionation is a versatile technique for separating and characterizing molecules and colloidal particles originally invented by J.C. Giddings. During operation, a mixture of molecules and/or particles is injected and are allowed to elute through the channel. Due to differences in hydrodynamic interactions, different component will have different retention in the channel, which gives rise to efficient separation. Elution time depends mainly on the translational diffusion coefficient of the component. Thus, the translational diffusion coefficient (henceforth: the diffusion coefficient) from a component can be estimated from the elution time.

For 'simple' FlFFF configurations (such as symmetrical channels and/or constant cross flow) the diffusion coefficient can be calculated using simple analytical expressions (Wahlund & Giddings, 1987; Litzén, 1993; Kirkland et al., 1992). However, most FlFFF experiments of today are run in more complex settings such as with asymmetrical channels and programmed cross flow since this has been shown to increase quality and decrease the time of analysis. In these cases it is not as straight forward to estimate the diffusion coefficient (or equivalently the hydrodynamic radius) from a measured retention time. FFFHydRad uses numerical methods to calculate diffusion coefficient from elution time. The technique is based on the method originally proposed by Nilsson et al. (2006).

An accurate measurement of the channel height is needed in the calculations but cannot easily be obtained from direct measurements due to compression and solvent effects. Channel height must therefore be obtained from a calibration experiments of a sample with known diffusion coefficient. FFHydRad includes functions for performing these calculations according to the technique described by Håkansson et al. (2012).

The diffusion coefficient can be used to calculate the hydrodynamic radius of a particle or molecule. Most molecules and particles are not spherical and it is often of interest to obtain some description of shape. FlFFF is often coupled to a Multi Angle Light Scattering (MALS) detector. The MALS detector will give information about a different length scale of the molecule (most often the rms-radius). Combining a MALS detector with elution time analysis thus gives two different length-scales of the molecule or particle under investigation. The ratio of length-scales has been used in order to obtain information about the geometrical shape.

A theoretical description of the algorithms used by FFFHydRad can be found in (Håkansson et al., 2012). A validation study can be found in (Magnusson et al., 2012)

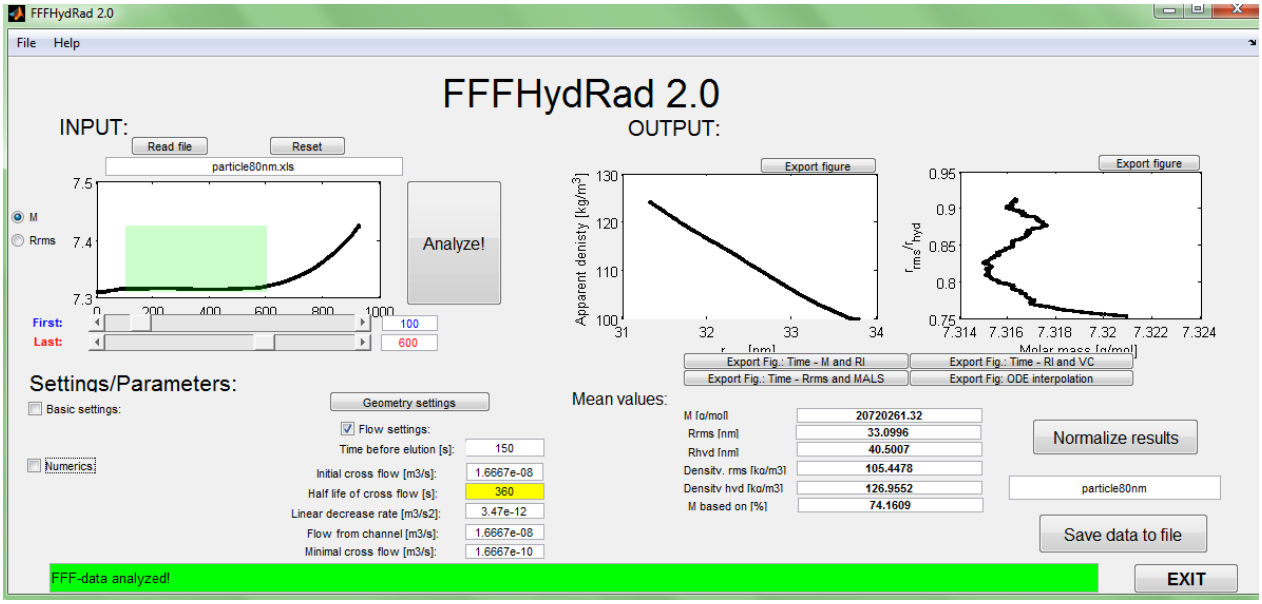
FFFHydRad is packed and distributed as a MATLAB app for ease of use and installation.

What is in this User Guide?

1. General information
2. Installation
3. Tutorials
4. Scientific comments

The main part of the user guide consists of the tutorials in Section 3, describing how to use the software for calculating channel height and analysing hydrodynamic radius. Note that the software was developed for use with FlFFF coupled to both RI and MALS detectors. A special tutorial is supplied in Section 3.2 for the user who only wants to calculate hydrodynamic radius from retention time without using these other detectors.

Screenshot


FFFHydRad 2.0
File Help
FFFHydRad 2.0
INPUT:
Read file
Reset
particle80nm.xls
M
Rrms
Analyze!
First:
Last:
100
600
OUTPUT:
Export figure
Apparent denisty [kg/m3]
Export figure
Export Fig.: Time - M and RI
Export Fig.: Time - RI and VC
Export Fig.: Time - Rrms and MALS
Export Fig: ODE interpolation
Settings/Parameters:
Basic settings:
Geometry settings
Flow settings:
Time before elution [s]: 150
Initial cross flow [m3/s]: 1.6667e-08
Half life of cross flow [s]: 360
Linear decrease rate [m3/s2]: 3.47e-12
Flow from channel [m3/s]: 1.6667e-08
Minimal cross flow [m3/s]: 1.6667e-10
Numerics:
Mean values:
M [g/mol] 20720261.32
Rrms [nm] 33.0996
Rhyd [nm] 40.5007
Density. rms [kg/m3] 105.4478
Density hyd [kg/m3] 126.9552
M based on [%] 74.1609
Normalize results
particle80nm
Save data to file
FFF-data analyzed!
EXIT

1. General information

1.1 License and copying

This software is free to download, install, use and modify for all applications if appropriately cited. Users are encouraged to cite the papers Nilsson et al. (2006) and Håkansson et al. (2012).

1.2 Version History

MAPLE-based version by Björn Bergenståhl and Lars Nilsson	2005
GUI-Beta 1 (for testing at Food Technology, Lund University)	2008
FFFHydRad 1.0 released	2009
FFFHydRad 1.3 released	2011
FFFHydRad 2.0 released	2013

1.3 New to version 2.0

- Complete rework of documentation
- Improved speed for reading result files.
- Support for reading data from xlsx-files.
- Users do not need MATLAB Statistic Toolbox.
- Converted to a MATLAB app.
- Output time is in the same scale as input time.
- Results saved to both txt and xls format.
- Rms- and hyd-based densities logically named in result files.

1.4 Required Software

FFFHydRad 2.0 is supplied as a MATLAB App and requires MathWorks MATLAB version 2012b or later. Versions working with earlier versions can be supplied.

1.5 Bugs, questions or suggestions

Reports of bugs, questions about the software, comments and enhancement suggestions could be sent to andreas@a-hakansson.se. Please include "FFFhydRad 2.0" in the subject line.

1.6 Developers

FFFhydRad has been developed by Andreas Håkansson, Lars Nilsson and Björn Bergenståhl, The Food Colloids Group, Lund University, Sweden.

2 Installing and running FFFHydRad 2.0

2.1 Installation

The software is supplied as an installation file *FFFHydRad.mlappinstall*. Download the file and open MATLAB. Click the 'APPS' tab (1) and then click 'Install App' (2), see Fig. 2.1.

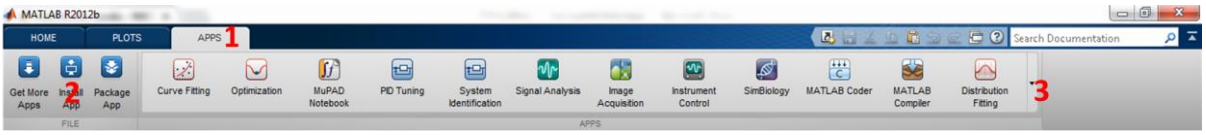


Figure 2.1. *Installing a MATLAB app.*

2.2 Opening FFFHydRad

To open FFFHydRad simply click on the 'APPS' list (1 in Fig. 2.1) and expand the list by clicking the down arrow (3 in Fig. 2.1). Locate FFFHydRad in the list and click on the icon.

3. Using FFFHydRad 2.0 - Tutorial

This section is a detailed tutorial on how to use the software for reading and analyzing data. A sample data file, *particle80nm.xls*, have is supplied with the installation and will be used in the tutorial. The data is from a validation experiment described in Magnusson et al. (2012).

3.1 Tutorial 1 - Using FFFhydRad with full dataset

Running FFFHydRad will open the main window, shown in Fig 3.1.

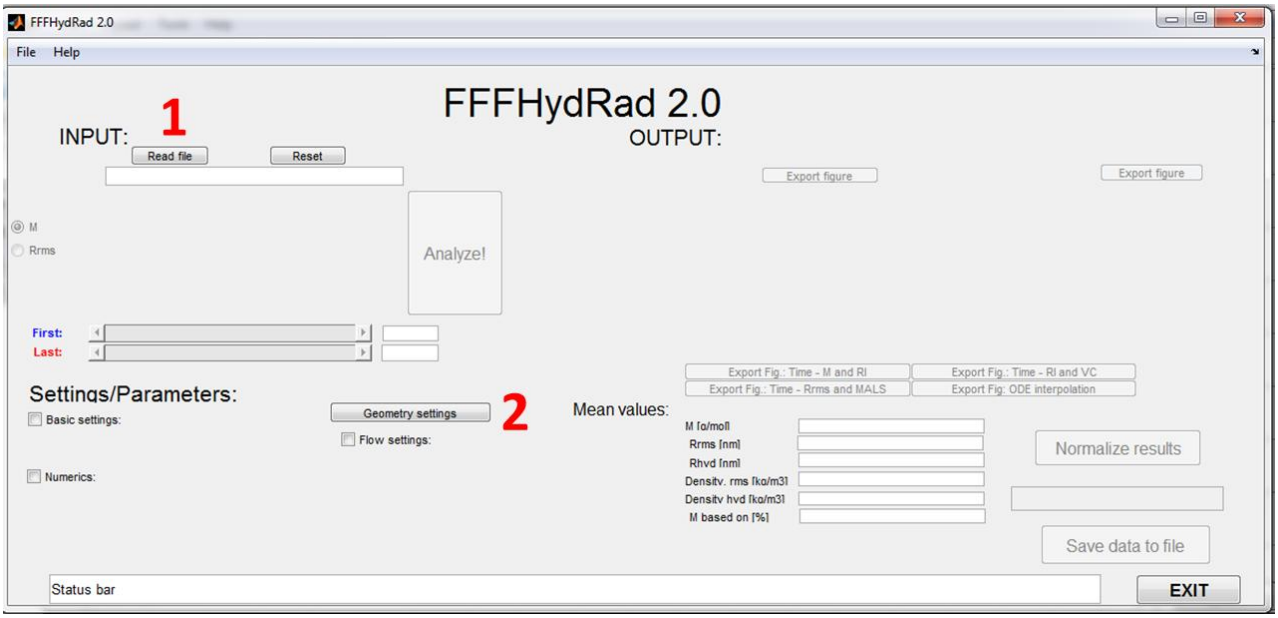


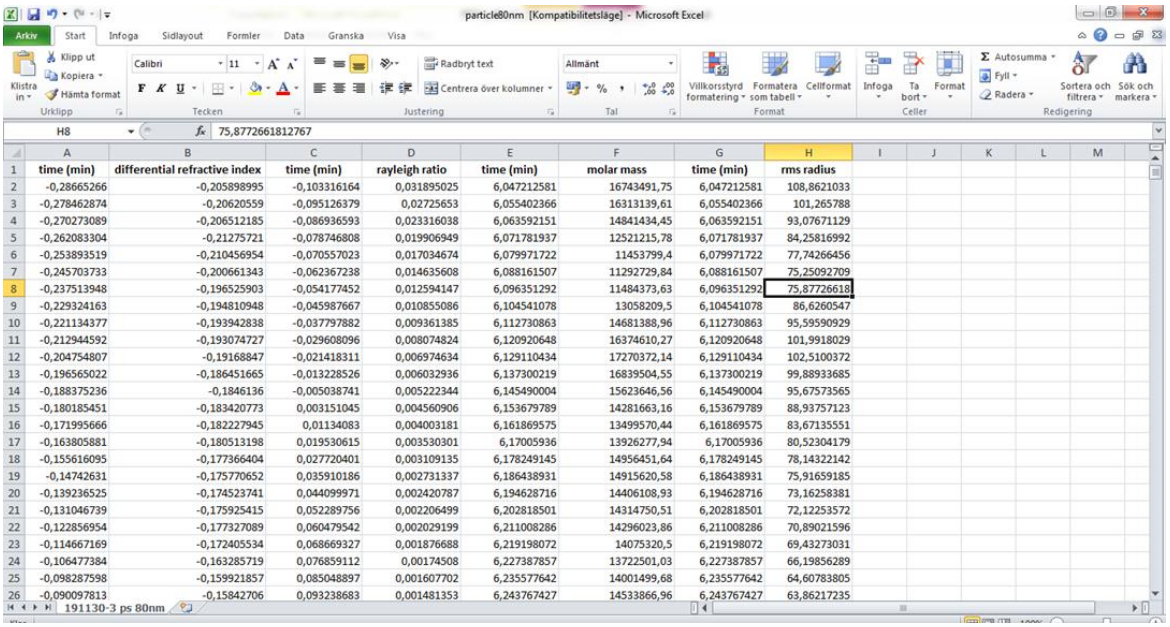
Figure 3.1. *The main window*

3.1.1 Reading data

The first step is to open a file containing FFF-data. Click the 'Read file' button (1 in Fig 3.1). This will open the standard file-open dialogue of your operating system. Select the appropriate Microsoft Excel file (software supports both .xls and .xlsx files) in the list and press OK. For this tutorial, pick *particle80nm.xls*.

The data in the xls-file must be arranged in a manner recognized by the software. (See Section 3.2 for information of how to use the software if you do not have all these detectors but only need to calculate diffusion coefficient corresponding to a given retention time.) The data should be arranged in eight columns as displayed in Fig 3.2. The first two columns are the time and the corresponding RI-readings. The next two columns are time and Rayleigh ratio (from MALS), after that time and molar mass and then finally time and rms-radius. As could be seen in Fig 3.2 the time vectors can differ, the program also allows for missing values in the rms Radius. (For more information on time scales see Section 3.3.)

In the sample file shown in Fig 3.2 the first row of the Excel sheet contains a text description, this is recommended in order to keep track on the columns, but optional for FFFHydRad, the software will skip text rows in reading the data.



	A	B	C	D	E	F	G	H	I	J	K	L	M
1	time (min)	differential refractive index	time (min)	rayleigh ratio	time (min)	molar mass	time (min)	rms radius					
2	-0,28665266	-0,205898995	-0,103316164	0,031895025	6,047212581	16743491,75	6,047212581	108,8621033					
3	-0,278462874	-0,20620559	-0,095126379	0,02725653	6,055402366	16313139,61	6,055402366	101,265788					
4	-0,270273089	-0,206512185	-0,086936593	0,023316038	6,063592151	14841434,45	6,063592151	93,07671129					
5	-0,262083304	-0,21275721	-0,078746808	0,019906949	6,071781937	12521215,78	6,071781937	84,25816992					
6	-0,253893519	-0,210456954	-0,070557023	0,017034674	6,079971722	11453799,4	6,079971722	77,74266456					
7	-0,245703733	-0,200661343	-0,062367238	0,014635608	6,088161507	11292729,84	6,088161507	75,25092709					
8	-0,237513948	-0,196525903	-0,054177452	0,012594147	6,096351292	11484373,63	6,096351292	75,87726618					
9	-0,229324163	-0,194810948	-0,045987667	0,010855086	6,104541078	13058209,5	6,104541078	86,6260547					
10	-0,221134377	-0,193942838	-0,037797882	0,009361385	6,112730863	14681388,96	6,112730863	95,59590929					
11	-0,212944592	-0,193074727	-0,029608096	0,008074824	6,120920648	16374610,27	6,120920648	101,9918029					
12	-0,204754807	-0,19168847	-0,021418311	0,006974634	6,129110434	17270372,14	6,129110434	102,5100372					
13	-0,196565022	-0,186451665	-0,013228526	0,006032936	6,137300219	16839504,55	6,137300219	99,88933685					
14	-0,188375236	-0,1846136	-0,005038741	0,005222344	6,145490004	15623646,56	6,145490004	95,67573565					
15	-0,180185451	-0,183420773	0,003151045	0,004560906	6,153679789	14281663,16	6,153679789	88,93757123					
16	-0,171995666	-0,182227945	0,01134083	0,004003181	6,161869575	13499570,44	6,161869575	83,67135551					
17	-0,163805881	-0,180513198	0,019530615	0,003530301	6,17005936	13926277,94	6,17005936	80,52304179					
18	-0,155616095	-0,177366404	0,027720401	0,003109135	6,178249145	14956451,64	6,178249145	78,14322142					
19	-0,14742631	-0,175770652	0,035910186	0,002731337	6,186438931	14915620,58	6,186438931	75,91659185					
20	-0,139236525	-0,174523741	0,044099971	0,002420787	6,194628716	14406108,93	6,194628716	73,16258381					
21	-0,131046739	-0,175925415	0,052289756	0,002206499	6,202818501	14314750,51	6,202818501	72,12253572					
22	-0,122856954	-0,177327089	0,060479542	0,002029199	6,211008286	14296023,86	6,211008286	70,89021596					
23	-0,114667169	-0,172405534	0,068669327	0,001876688	6,219198072	14075320,5	6,219198072	69,43273031					
24	-0,106477384	-0,163285719	0,076859112	0,00174508	6,227387857	13722501,03	6,227387857	66,19856289					
25	-0,098287598	-0,159921857	0,085048897	0,001607702	6,235577642	14001499,68	6,235577642	64,60783805					
26	-0,090097813	-0,15842706	0,093238683	0,001481353	6,243767427	14533866,96	6,243767427	63,86217235					

Figure 3.2. *A sample data file.*

If a correctly formatted xls-file has been chosen in the open dialogue (1 in Fig. 3.1), after a few seconds, the logarithm of molecular mass (in g/mol) as a function of time position is shown in the main window (1 in Fig 3.3). Above the graph (2 in Fig 3.3) the name of the chosen file could be seen. Also the status bar at the bottom of the window turns yellow (3 in Fig. 3.3) and the complete path to the chosen file is displayed.

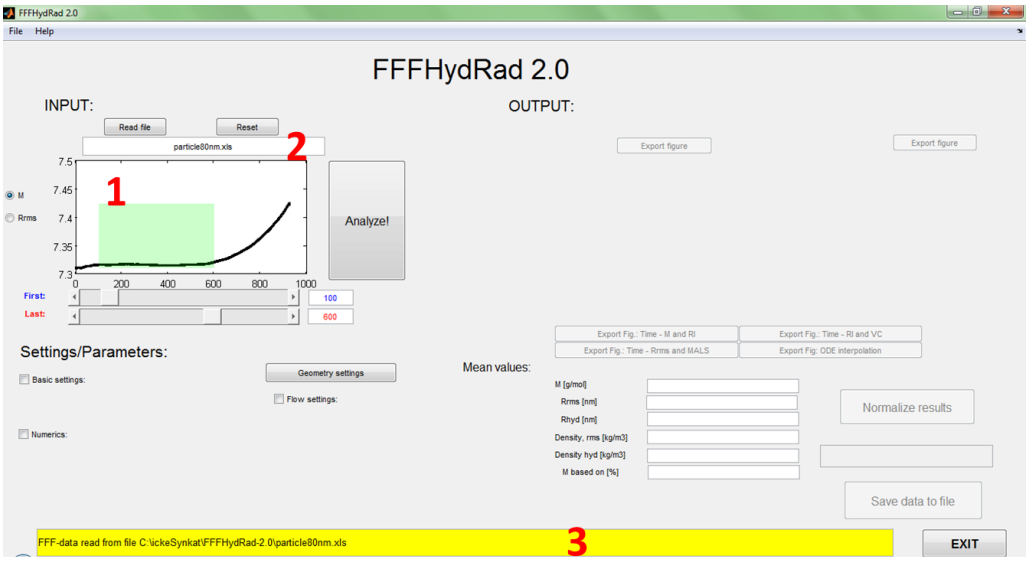


Figure 3.3. *Open data*.

3.1.2 Calculating channel height

Before analysis could be performed, the correct channel geometry must be specified and the channel height must be calculated from a calibration experiment. To do so, click 'Geometry settings' (2 in Fig. 3.1). This opens the Geometry settings window (see Fig. 3.4). Use the edit boxes (1 in Fig 3.4) to specify the geometry of the channel. A schematic image of the channel has been inserted (2 in Fig. 3.4) in order to help the user.

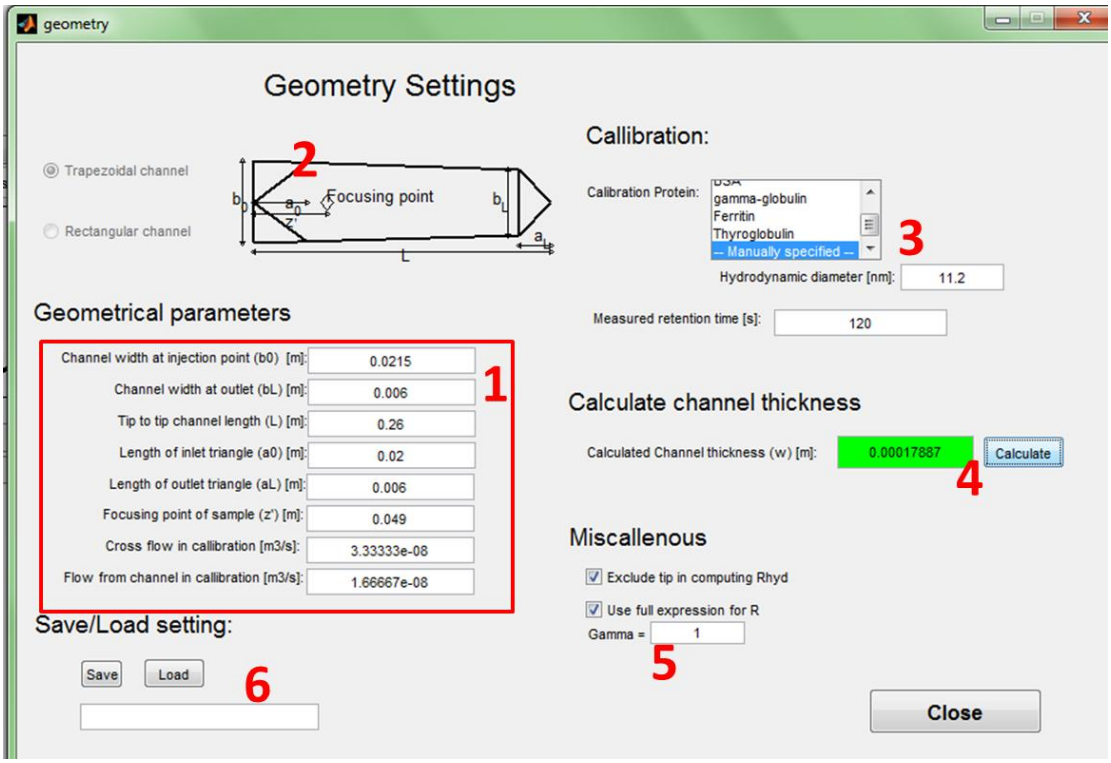


Figure 3.4. *Geometry setting.*

Next, specify the hydrodynamic radius of the calibration particle used. Values for a number of common calibration proteins has been inserted in the list (3 in Fig. 3.4) and can be chosen by clicking. However, since these values has been calculated from tabulated diffusion coefficient (see table 3.1) they depend on factors such as temperature, solvent and accuracy in the tabulated values. A recommendation is to always chose "--Manually specified--" and put the known hydrodynamic radius of the calibration particle (in nm) in the edit-box.

Next, put the measured retention time (in seconds) of the calibration particle in the box below.

When changes are made to the settings, the *w*-box (4 in Fig. 3.4) turns red to indicate that the calculations have not been updated. Press 'Calculate' (4 in Fig. 3.4) to update the value.

FFFHydRad allows for different degrees of completeness in the theoretical expression for the retention equations. Many studies neglect the retention from the small tip of the channel; tip retention can be excluded or included from the calculations by clicking a tick-box (5 in Fig. 3.4). Two different expressions for the retention parameter *R* are also available. The full expression refers to the expression derived by Giddings et al. (1987) including excluded volume effects. Including the full expression is highly recommended (see Håkansson et al., 2012 for a comparison, the full expression corresponds to IV in table 1 of the reference).

It is possible to save and load channel settings for reuse(6 in Fig. 3.4).

Click the 'Close' button when you are done with the calculation (make sure that the *w*-field is green indicating that the calculations have been updated after the last changes).

Table 3.1. *Some protein standards for channel calibrating.*

Protein	Hydrodynamic diameter in PBS at 25 °C [nm]
Ovalbumin	5.2
BSA	6.6
Gamma-globulin	10.1
Ferritin	11.2
Thyroglobulin	15.5

Data from Schimpf et al. (2000).

3.1.3 Choosing interval

If we now return to the main window (Fig. 3.5). A plot of the data showing either the logarithm of molar mass (M) or the rms-radius in nm is displayed upon loading data. Note that the x-axis do not refer to time but to the numbering of time-points in the experimental data (*i.e.* 106 refers to the 106:th sampling time of the detector).

Sliders (1 in Fig. 3.5) and edit boxes (2 in Fig. 3.5) allows the user to choose what part of the data to include in the analysis. The current position of the selected interval is displayed in the graph by the green rectangle. (The rectangle turns red if errors in the interval specifications are found.)

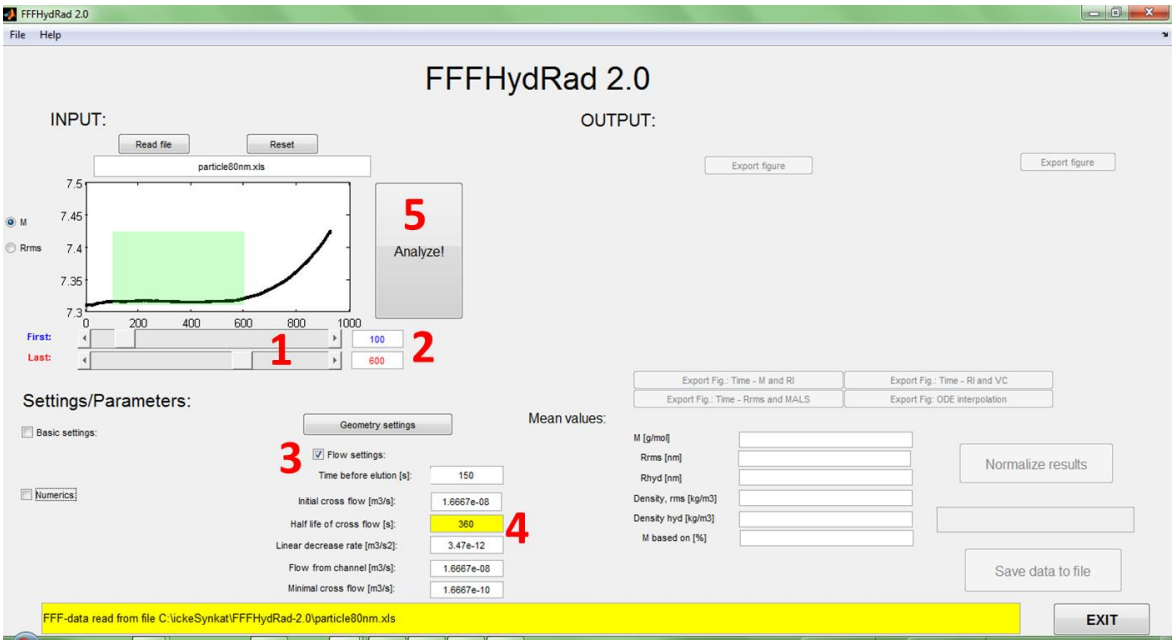


Figure 3.5. *Choosing the analysis interval and altering flow settings.*

3.1.4 Flow setting

Ticking the box entitled 'Flow settings' (3 in Fig. 3.5) displays a number of new settings. Chose time before elution (see Section 3.3), initial cross flow, flow rate from channel. If using a decreasing cross-flow it is also advised to enter the minimal cross flow allowed by the apparatus. Note the SI units!

FFFHydRad allows for decreasing cross-flows, either from exponential decrease or linear decrease:

<u>Exponential decaying cross-flow</u>

Enter the half time of the cross-flow flow-rate (in seconds) in the box and press the enter key. The box turns yellow to indicate that you are using exponential decay.

Linear decaying cross-flow
Enter the decay rate in the appropriate box and press enter. The yellow marking moves to the linear decrease box to display that the analysis is performed with linear decay.

Constant cross-flow
Put a very large number or inf (MATLAB-notation for infinity) in the 'Exponential decay half time' box and press enter.

Once satisfied with the time interval, click 'Analyze!' (5 in Fig 3.5) to proceed. Analysing the data will take a few seconds, depending on your computer and operating system. Once analysis is complete a summary of the results will be displayed in the MATLAB command window.

3.1.5 Displaying and saving the results

When completed, the status bar at the bottom of the screen will turn green. Resulting statistics of the selected data range will be shown in a list (1 in Fig 3.6) together with a graph of apparent density over rms-radius (3 in Fig 3.6) and radius-quotient over Molar Mass (4 in Fig 3.6). However, before looking at the results, it is highly recommended to have a look at the interpolation underlying the estimations. Press the 'Export: ODE interpolation' button (2 in Fig. 3.6). This opens a plot showing the numerical solution to the ode and interpolation. Make sure that the interpolations fits well to the ode solution (see Fig. 3.7). If there is a bad fit (can be seen by a very jumpy 'saw-tooth'-looking interpolation, try decreasing the Imax-setting under the Numeric settings until it looks smooth as Fig. 3.7).

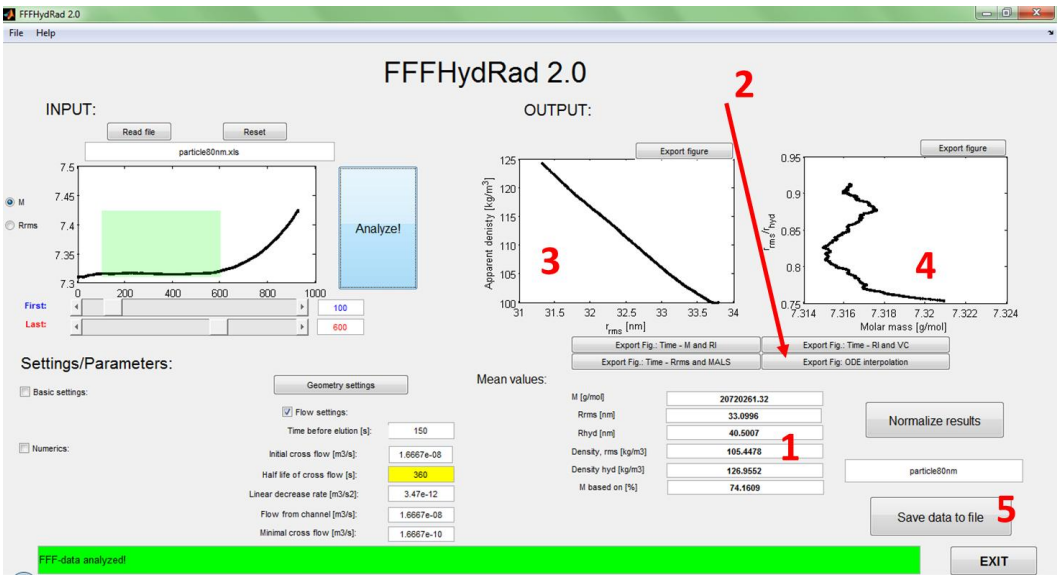


Figure 3.6. *Displaying the results.*

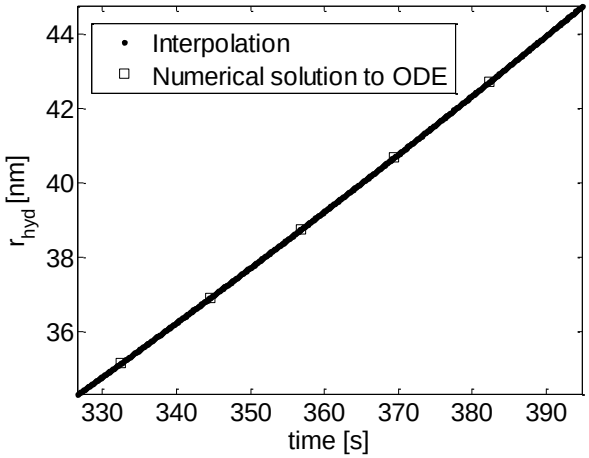


Figure 3.7. A good *ODE Interpolation.*

From Fig. 3.6 it can be seen that the (mass weighted) hydrodynamic radius of the sample is 40.50 nm. The z-weighted value can be seen in the command prompt of MATLAB after each evaluation together with some additional statistics. DLS experiments indicate a diameter of 87.4 nm which fit rather well with the predicted result.

The rms-hydrodynamic radius ratio can also be seen in the results. For a spherical particle the ratio should be close to 0.77. The experimental results indicated a ratio in the interval 0.77 to 0.9. Variations seems unsystematic indicating a uniform shape across the investigated distribution.

Each graph has an "export"-button in the top right corner. Pushing this button will draw the graph in a new window, here it could also be edited and exported to different image formats by MATLABs standard utilities.

Results can be saved to txt and xls files by pressing the save button (5 in Fig. 3.6). Two new text files will be created in the same directory as where the xls-file was read. The first file contains the calculated hydrodynamic radius together with the supplied data and some statistical information. This file has the same name as the xls-file but with "_analyzed" added at the end. The second file contains all settings used in calculating the results and has the extension "_settings". If the files already exist in your directory you will be asked if you want to overwrite them. An xls-file with '_results' added to the original file-name will also be created.

The names of the newly created files together with paths will be shown in the green status bar at the bottom of the screen when the files have been correctly saved.

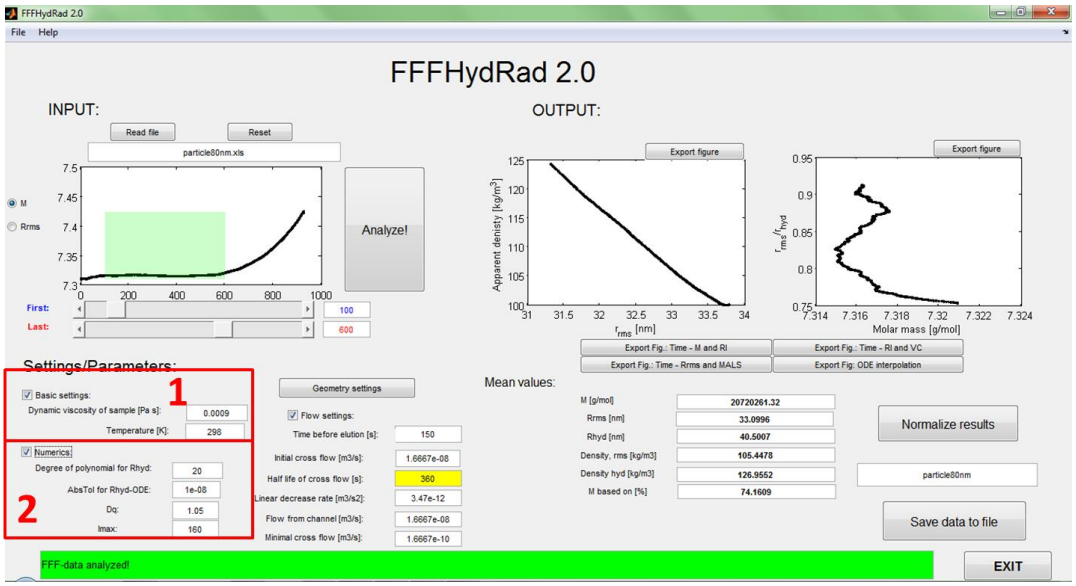


Figure 3.8. *More settings*.

3.1.6 More settings

Additional settings are available after clicking the 'Basic settings' (1 in fig 3.8) or 'Numerics' (2 in Fig. 3.8) check-boxes. Basic settings include dynamic viscosity of the carrier liquid and experiment temperature.

FFFHydRad works by solving the retention equation for a large number of particle sizes, i.e. the retention time for a large number of hydrodynamic radii are calculated. An interpolation is needed in order to translate this to values for all the experimental times. The first numeric option is the type of translation scheme. The recommended setting is '20' which corresponds to a piecewise linear interpolation. For historical reasons, the software also allows for fitting the solution to a polynomial and evaluating the polynomial for intermediate values. Different degree polynomials can be chosen by choosing degrees between 2-9.

The setting 'D0' is the ration between two succeeding simulated diffusion coefficient, decreasing this number towards 1 increase the computational load and the resolution. 'Imax' is the total number of simulated sizes. Always make sure that the interpolation fits the simulated data after analysing the date with new numeric settings (compare with Fig. 3.7).

3.1.7 Resetting / Closing FFFHydRad

In order to reset the program, clear results and loaded files, press 'Reset' in the upper left corner in the main window. Note that settings are kept.
Close the program by pressing 'Exit' in the lower right of the main window or use the menus File->Exit.

3.2 Tutorial 2 - Using FFFhydRad with a reduced dataset

FFFHydRad can be used if using other detectors than the ones described in Section 3.1.

3.2.1 Using a template Excel file

Start by opening the reducedTemplate.xls-file. The fields that need to be altered by the user are marked in yellow. First put whichever signal is used to calculate concentration and the corresponding time in the columns marked 1 in Fig. 3.9. Then insert the times for which hydrodynamic radius needs to be obtained in the column marked 2 in Fig. 3.9 (note that multiple values are needed, see Section 3.2.2 if data is needed for single time-points). Make

sure the columns marked 2 and all columns to the right of it have the same number of rows with data and that the columns in 1 and the columns to the left of them all have the same number of rows with data. Do not forget to save the file with a new name before closing.

Now, run the analysis as described in Section 3.1 and save results to file. Open the xls-file created by the software to see the results.

3.2.2 Manual estimation of single time points (peak values)

When only hydrodynamic radius corresponding to a single time-point is needed (e.g. for peak value), a MATLAB-function 'manualRhyd.m' has been supplied. First runt an analysis with FFFHydRad using a sample Excel-file such as particle80nm.xls. The actual data in the file is not needed but make sure to fill in all geometrical and flow settings correctly in the FFFHydRad-windows. Hit 'Analyze' and exit FFFHydRad.

Now in the MATLAB Command Window type:

```
load interm;
manualRhyd(timeIwantRfor,1,utStruct);
```

while substituting **timeIwantRfor** with the time (in seconds) for which the hydrodynamic radius is needed. Time should be given in the same time scale as the data in the Excel-file, see Fig. 3.10.

Example: The peak in Fig. 3.10 is located at 8.8 min (=528 s).

```
load interm;
manualRhyd(528,1,utStruct);
```

will return

```
At t = 528 s, Rhyd = 42.039 nm.
```

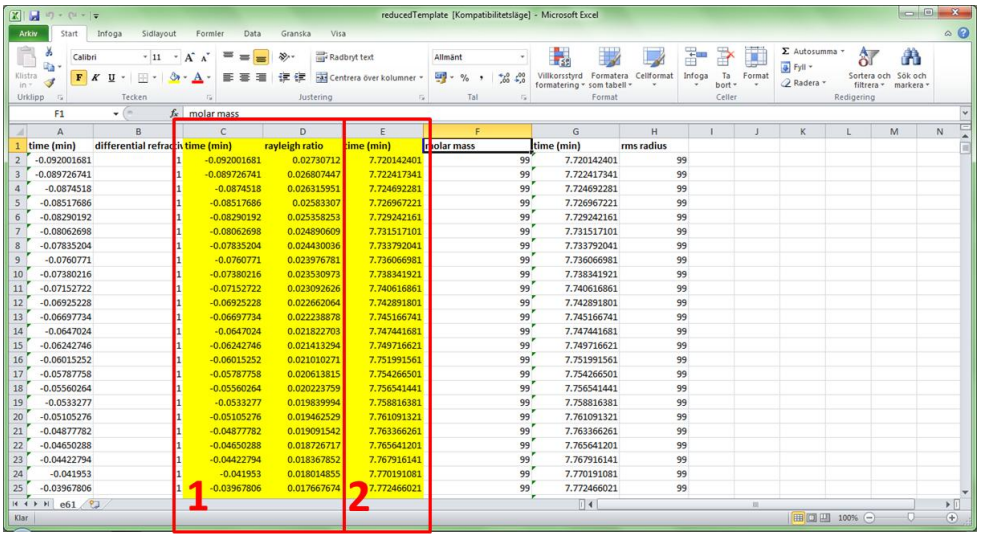


Figure 3.9. *reducedTemplate.xls.*

3.3 A Note on time

As seen in Fig. 3.2, the Excel file used as an input to the software allows the user to use different time vectors for different detectors. Fig. 3.10 displays the Rayleigh ratio (in black) and r_{rms}-radius (in red) from the input file particle80nm.xls. Here the concentration signal

covers all of the experiment whereas the r_{rms}-signal was only captured where the material actually eluted.

Also note that the concentration detector was switched on before starting elution. The time before elution (t_{be} in Fig. 3.10) must be given in the 'Flow Settings' (3 in Fig. 3.5) for accurate determination. Fig. 3.10 also shows the void time (t_0) to clarify the difference, the void time is calculated in the software from membrane geometry and channel height, it does not need to be supplied by the user.

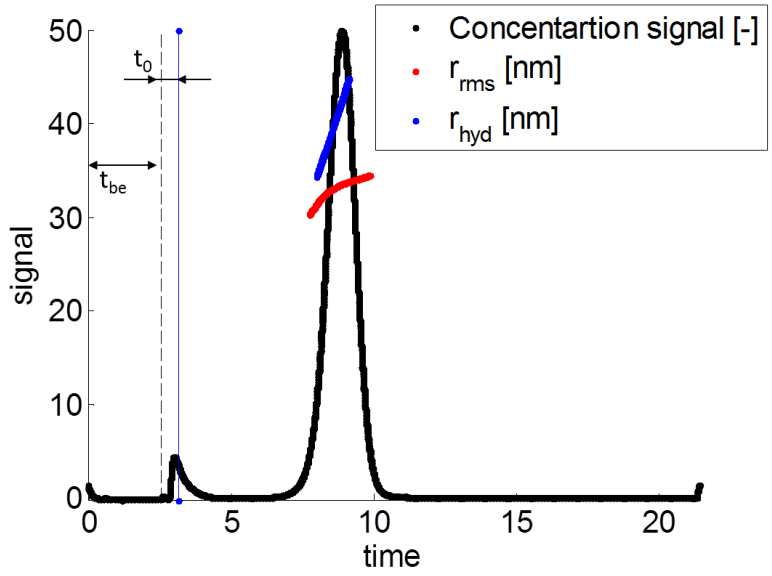


Figure 3.10. *Illustration of how to treat elution time in the evaluations.*

References

Håkansson, A., Magnusson, E., Bergenståhl, B., Nilsson, L. (2012). *Hydrodynamic radius determination with asymmetrical flow field-flow fractionation using decaying cross-flow. Part I. A theoretical approach.* Journal of Chromatography A 1253, 120-126.

Kirkland J., Dilks, C., Rementer S., Yau, W., (1992). *Asymmetric-channel flow field-flow fractionation with exponential force-field programming*. Journal of Chromatography, 593, 339-355.

Nilsson, L., Leeman, M., Wahlund, K.-G., Bergenståhl, B., (2006). *Mechanical Degradation and Changes in Conformation of Hydrophobically Modified Starch.* Biomacromolecules, 7, 2671-2679.

Li, P.; Hansen, M. In Field-Flow Fractionation Handbook; Schimpf, M. E., Caldwell, K., Giddings, J. C., Eds.; John Wiley & Sons Inc: New York, 2000.

Litzén, Anne, (1993). *Separation Speed, Retention, and Dispersion in Asymmetrical Flow Field-Flow Fractionation as Functions of Channel Dimensions and Flow Rates*. Anal. Chem., 65, 461-470

Magnusson, E., Håkansson, A., Janiak, J., Bergenståhl, B., Nilsson, L. (2012). *Hydrodynamic radius determination with asymmetrical flow field-flow fractionation using decaying cross-flow. Part II. Experimental evaluation*. Journal of Chromatography A 1253, 127-133.

Wahlund, K.-G., Giddings J., (1987). *Properties of an asymmetrical flow field-flow fractionation channel having one permeable wall*. Analytical Chemistry, 59, 1332-1339.

Schimpf, M.E., Cladwell, K., Giddings, J.C. (2000). *Field-Flow Fractionation Handbook*. Wiley.