Rapid Kinetics and Spectroscopy instruments - Technical note #38

Anisotropy T- format using MOS-250 and two additional channels

The aim of this note is to provide a step by step procedure to perform anisotropy measurements using T-format configuration. To illustrate this note the refolding kinetics of hen egg lysosyme is performed. Control of syringes, acquisition and analysis are made using Bio-Kine version 4.01. The user is supposed to know how to operate MOS-250, PMS-250 and Bio-Kine.

EXPERIMENTAL CONDITIONS

- ➢ **MOS-250** equipped with 150W Xenon mercury lamp.
    - Excitation wavelength: 297 nm.
    - Bandpass: 10 nm
    - Detection using 2 PMS-250 tubes (Pm tubes equipped with 320 nm cut-off filters)
- ➢ **SFM-20** equipped with 10 ml syringes.
    - Syringe 1: 1.5 mg/ml hen egg lysosyme in 6M Guanidine Hydrochloride
    - Syringe 2: water
    - Mixing ratio: 1/8 (lysosyme/water)
    - Cuvette: FC-20.
    - Mixer: Berger Ball
    - Total flow rate: 3.8 ml/s

Remark: the aim of this note is not to reach the shortest dead time but to perform anisotropy measurement

DESCRIPTION OF THE SYSTEM

In fact **MOS-250** is used at a light source and to collect signals coming from both PM tubes. Two **PMS-250** are required to perform anisotropy measurement. Both PM tube are installed at 90° to the excitation light and are equipped with a polarizer, the position is set by the user (see further). A polarizer is also installed between the optical fiber and the cuvette; the position of the polarizer is also set by the user (see further). Signals measured by both PMS-250 are transferred to the **acquisition board** via the **MOS-250 external inputs**. A general scheme of the system is given in figure 1.

Excitation polarizer
Depending on the orientation of the polarizer, vertical or horizontal polarized light is produced. The orientation is indicated using numbers (1 to 4), in standard configuration positions 1 and 3 are used to polarize vertically, 2 and 4 to polarize horizontally. If for a reason the polarizer is removed and installed back the position indicated above may be inverted. To change orientation of the polarizer, turn the labeled wheel.

Emission polarizers
The **emission polarizer** is mounted inside the PM tube cap. The polarizer is inserted between a **cut-off filter** (also installed into the PM tube and separated by O-ring foam) and a **collimator with lens** which is directly installed onto the stopped-flow observation head (refer to figure 2).
A **brace** is used to swap orientation of the polarizer from vertical to horizontal using a 90° rotation. The **polarizer lock screw** (see figure 2) is used to lock the brace to the PM tube when the orientation of the polarizer is set (see the procedure further). The **PM lock screw** is used to lock the PM tube and thus to avoid any moves during the shots.

<u>**Warning**</u>**: Use the brace to swap orientation of polarizer. Do not change polarization by turning the PM tube itself because it can be turned to more than 90° and the setting can be lost!**
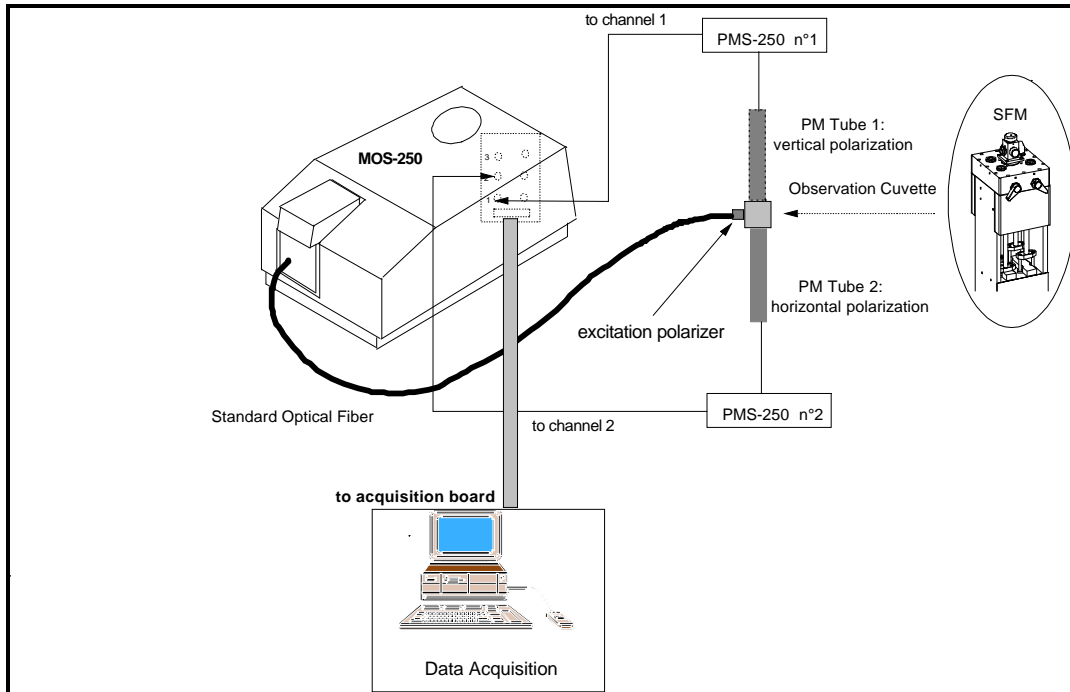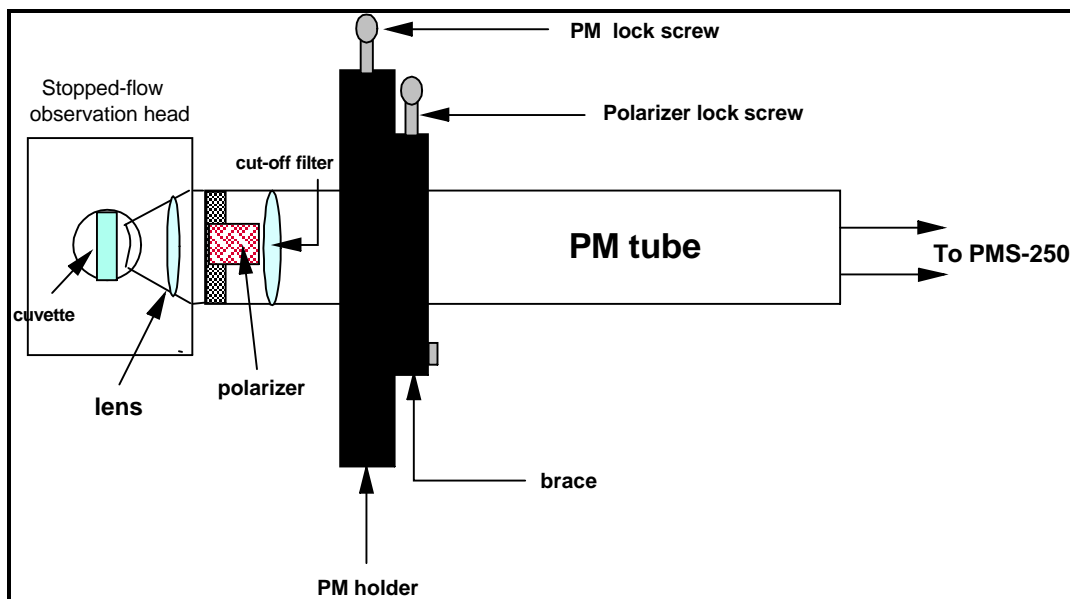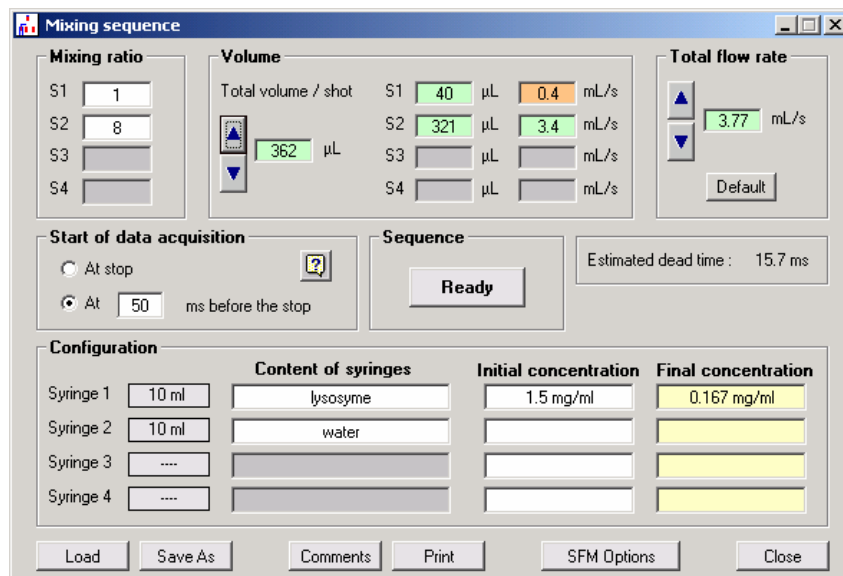
Figure 1 : general scheme

Figure 2 : scheme of the emission configuration.

PREPARING THE SYSTEM:

➢ **MOS-250:**
- Connect MOS-250 to PC via serial port.
- Connect MOS-250 to acquisition board using dedicated cable.

➢ **PMS-250 n°1 (combined with vertical polarization):**
- Install a 320 nm cut-off filter in the PM tube.
- Install O-ring foam on the filter to avoid any move.
- Close the PM tube with the special cap equipped with polarizer.

- Connect the PM tube to PMS-250 n°1 (2 cables)
- Connect the '**PM out filtered**' output of PMS-250 to **external channel1 input** of MOS-250.
- Plug the PMS-250.
➢ **PMS-250 n°2 (combined with horizontal polarization):**
  - Install a 320 nm cut-off filter in the PM tube.
  - Install O-ring foam on the filter to avoid any move.
  - Close the PM tube with the special cap equipped with polarizer.
  - Connect the PM tube to PMS-250 n°2 (2 cables)
  - Connect the '**PM out filtered**' output of PMS-250 to **external channel2 input** of MOS-250.
  - Plug the PMS-250.
➢ **MPS**
  - Connect the stopped-flow device.
  - Connect the hard-stop.
  - Connect the serial port.
  - Plug the MPS.
➢ **Configuration of Bio-Kine:**
  - Load Bio-Kine.
  - Enter the **Install, device** menu and select the stopped-flow device and its serial port.
  - Select MOS-250 device and its serial port.
  - Select the SFM-20 device and 10 ml syringes.
  - Initialize the syringes, and then fill the syringes.
  - Open the transient recorder window.
  - Click on the Advanced button, then on the SFM Options button: select the FC-20 cuvette and a 2 ms lead time. Validate by clicking the OK button.
  - Edit the stopped-flow sequence shown in figure 3.
  - Run few shots to fill the cuvette with final solution (without acquisition). This final solution is used to set orientation of polarizers and high voltage adjustment.

Figure 3: stopped-flow sequence

The system is now physically ready; the optical settings can be done

DESCRIPTION OF THE METHOD USED

- To perform anisotropy 4 signals are necessary:
    I_{vv} : vertical excitation, signal measured with PMS-250 polarized vertically
    I_{vh} : vertical excitation, signal measured with PMS-250 polarized horizontally
    I_{hv} : horizontal excitation, signal measured with PMS-250 polarized vertically
    I_{hv} : horizontal excitation, signal measured with PMS-250 polarized horizontally

- The acquisition is done in two phases: first the measure of the **G factor** and then the real measurement.

- The G factor is determined using horizontal excitation and is given by **$G = I_{hh}/I_{hv}$**.

- The real measurement is done with vertical excitation, PMS-250 simultaneously measure **I_{vv}** and **I_{vh}**. Anisotropy and fluorescence can then be calculated using these parameters in the following equations

$A = (I_{vv} - G*I_{vh}) / (I_{vv} + 2*G*I_{vh})$ and **$F = I_{vv} + 2*G*I_{vh}$**

PM tube n°1: Determination of emission polarizer orientation

- **Excitation: vertical polarization** (position 3 for example)
- This setting is made in **diffusion mode** with a **360 nm excitation wavelength** (diffusion mode: the excitation wavelength is higher than the cut-off wavelength).
- Unscrew slightly **PM and polarizer lock screws**.
- Turn the **brace** in order to have the **polarizer lock screw** towards the user. By convention this position will correspond to the horizontal polarization (the vertical polarization is reached by a 90° rotation of the brace).
- Switch the PM on. On the **PMS-250 n°1** select a High voltage to get a response of the PM, for example adjust High voltage to get 2 Volts response.
- With both lock screws free, **turn the PM tube manually to find the position which gives the minimum response**. **This position corresponds to a horizontal polarization**. When this position found, turn the polarizer lock screw and read the PM response (V_1min_1). Using the brace, go to the vertical position and read the PM response (V_1max_1). Because of its geometry, the polarizer has 2 minima and maxima, so repeat the above procedure to find (V_1min_2).and (V_1max_2). **The best orientation is the one which gives the higher ratio (Vmax/Vmin)**.
In the example described:
$$V_1min_1 = 0.207 \text{ V and } V_1max_1 = 2.935 \text{ V}.$$
$$V_1min_2 = 0.350 \text{ V and } V_1max_2 = 2.508 \text{ V}.$$
The first set of values is selected. **The polarizer lock screw is blocked and should not be touch anymore.**
- Turn the brace to the vertical polarization (opposite to the users by convention). The response given by PM is 2.935 Volts (as determined before).
- Block the PM lock screw.
- **The first emission polarizer is now well oriented**.

PM tube n°2: Determination of emission polarizer orientation

• **Excitation: vertical polarization** (still on position 3)

• This setting is made in **diffusion mode** with a **360 nm excitation wavelength** (diffusion mode: the excitation wavelength is higher than the cut-off wavelength).

• Unscrew slightly **PM and polarizer lock screws**.

• Turn the **brace** in order to have the **polarizer lock screw** towards the user. By convention this position will correspond to the horizontal polarization (the vertical polarization is reached by a 90° rotation of the brace).

• Switch the PM on. On the **PMS-250 n°2** select a High voltage to get a response of the PM, for example adjust High voltage to get 3 Volts response for example.

• With both lock screws free, **turn the PM tube manually to find the position which gives the minimum response**. **This position corresponds to a horizontal polarization**. When this position found, turn the polarizer lock screw and read the PM response (V_2min_1). Using the brace, go to the vertical position and read the PM response (V_2max_1). Because of its geometry, the polarizer has 2 minima and maxima, so repeat the above procedure to find (V_2min_2).and (V_2max_2). **The best orientation is the one which gives the higher ratio (Vmax/Vmin)**.
In the example described:
$$V_2min_1 = 0.336 \text{ V} \text{ and } V_2max_1 = 5.600 \text{ V}.$$
$$V_2min_2 = 0.470 \text{ V} \text{ and } V_2max_2 = 2.670 \text{ V}.$$
The first set of values is selected. **The polarizer lock screw is blocked and should not be touch anymore.**

• Turn the brace to the vertical polarization (opposite to the users by convention). The response given by PM is 5.6 Volts (as determined before).

• Block the PM lock screw.

• **The second emission polarizer is now well oriented**.

ORIENTATION OF EXCITATION POLARIZER

Because of its geometry the excitation polarizer also has 2 'vertical polarized' positions and two horizontal ones. The aim of this section is to find the best 'vertical position' (1 or 3) and the best 'horizontal one' (2 or 4). The best vertical position is found by looking at the highest ratio V_1/V_2. The best horizontal position is found by looking at the lowest ratio V_1/V_2.

• **Excitation: vertical polarization** (still on **position 3**).

• The **emission polarizers** set before are '**vertically oriented'**.

• Set the **excitation wavelength to 297 nm** (experimental wavelength).

• Set the High voltage on both PMS-250 to get about 5 volts response on both channels (gain similar) (HV_1 =632 volts and HV_2 = 647 volts)

• Unlock **PM lock screw n°2** and turn the brace to the horizontal orientation. **Now, PM1 is vertically polarized and PM2 horizontally polarized**.

• Read the response of both PM for excitation position 3 : **V_1=5.3 Volts** and **V_2=2.9 volts.**

• **Change** the position of **excitation polarizer to position 1**.

• Read the response of both PM for excitation position 1 : **V_1=3.1 Volts** and **V_2=2.3 volts.**
The best position is the one giving the highest ratio (**V_1/V_2**)**.**

• **Position 3 is selected to produce vertical polarization at excitation**.

• **Change** the position of **excitation polarizer to position 2 (horizontal polarization)**.

• Read the response of both PM for excitation position 2 : **V_1=2.4 Volts** and **V_2=2.7 volts.**

• **Change** the position of **excitation polarizer to position 4 (horizontal polarization)**.

• Read the response of both PM for excitation position 4 : **V_1=2.5 Volts** and **V_2=3.5 volts.**

• Ratio V_1/V_2 is lower for position 2 so **position 2 is chosen to produce horizontal polarization**.

Vertical polarization is done in position 3 and horizontal polarization in position 2.

DETERMINATION OF THE G FACTOR

The polarizers are now well oriented, so depending on the status of the excitation signal $I_{vv}, I_{vh}, I_{hh}, I_{hv}$ are read on PMS-250.

- **Excitation**: **horizontal polarization** (position 2 as determined before).
- PM n°1 is still vertically polarized and PM n°2 horizontally polarized.
- The response given by PM are I_{hv}= 2.4 volts and I_{hh} =2.7 volts.
- Adjust the voltage applied on PM n°2 to get about the same response than PM n°1: this is done to have similar gain between the PMS-250.

$I_{hv} \approx I_{hh} \approx$ **2.4 volts** (which corresponds to **HV$_1$=632 volts** and **HV$_2$ = 630 volts**).

- In Bio-Kine, click on the Acquisition Setup button to load the acquisition parameters window.
- **Select channels 3 and 4** (these channels correspond to external inputs 1 and 2 for the acquisition board). Select **anisotropy T-format** acquisition mode.
- Click on the G factor 'Find/Stop' button to instantaneously calculate the G factor using **G= I_{hh}/I_{hv}**. Click a second time on this button to stop the calculation. Read the value obtained. **G =1.044** (never far from 1). The value obtained is kept in memory and will be used for later anisotropy and fluorescence calculations.

BACKGROUND CORRECTION

- **Excitation: vertical polarization** (position 3 as determined before).
- PM n°1 is still vertically polarized and PM n°2 horizontally polarized.
- Push some water into the cuvette directly from MPS unit.
- Still in the Acquisition Setup menu, determine the **background correction** factors by clicking on the **'Find/stop'** button. Click a second time on this button to stop the instantaneous measurement.
- In this example, **I°$_{vv}$= 2.531** and **I°$_{vh}$= 0.294 volts.**
- This parameters are taken into account in the software for anisotropy and fluorescence calculation. The G factor is only applied on the horizontal component.

ACQUISITION PARAMETERS

- **Excitation: vertical polarization** (position 3 as determined before).
- PM n°1 is still vertically polarized and PM n°2 horizontally polarized.
- Still in the Acquisition Setup menu:
- The **G factor** is now determined.
- The **background correction** factors are determined.
- Select the **A/F** option to follow anisotropy and fluorescence (it is also possible to follow I_{vv} and I_{vh})
- Select the **trigger**.
- Select a 1 ms sampling period, and a 2 s total time for the acquisition.
- Validate by clicking OK.
- On the PMS-250 choose an **output filter in accordance with the sampling rate selected for the acquisition** (**1 ms on both PMS-250**)

The system is now totally ready for shots.

RESULTS

- An accumulation of 16 shots is done, results are presented in figure 4 (anisotropy result) and in figure 5 (fluorescence result).
- The fluorescence kinetics is fitted with a double exponential **k$_1$ =40 s^{-1} and k$_2$ =4.2 s^{-1}**
- The anisotropy kinetics is fitted with a single exponential **k$_1$ =32 s^{-1}.**

(**I_{vv}** and **I_{vh}** can be reached in the analysis menu by choosing **Tools, anisotropy, T-format** in the main menu).
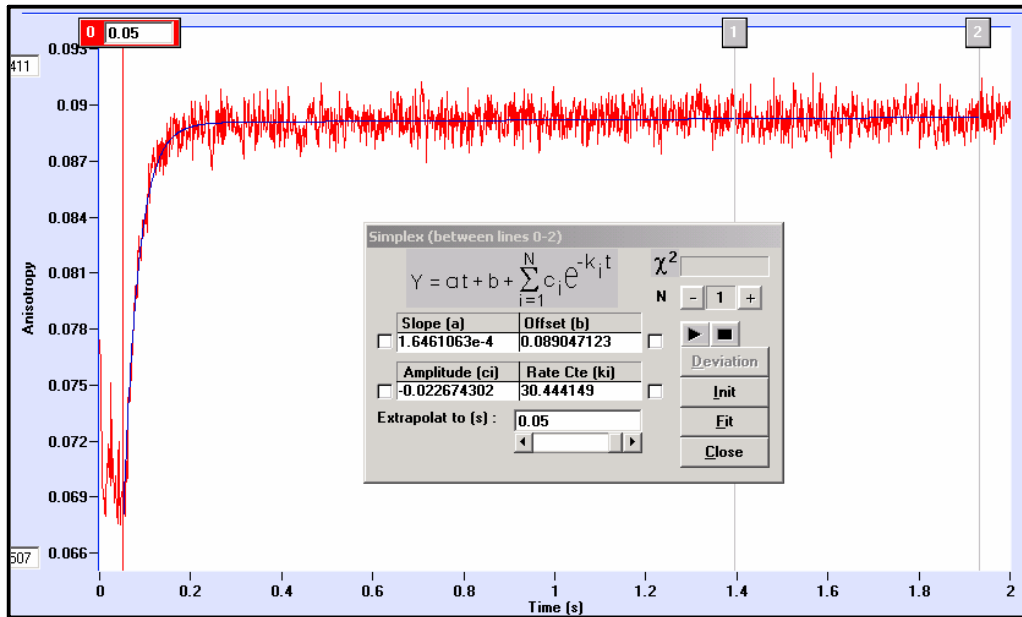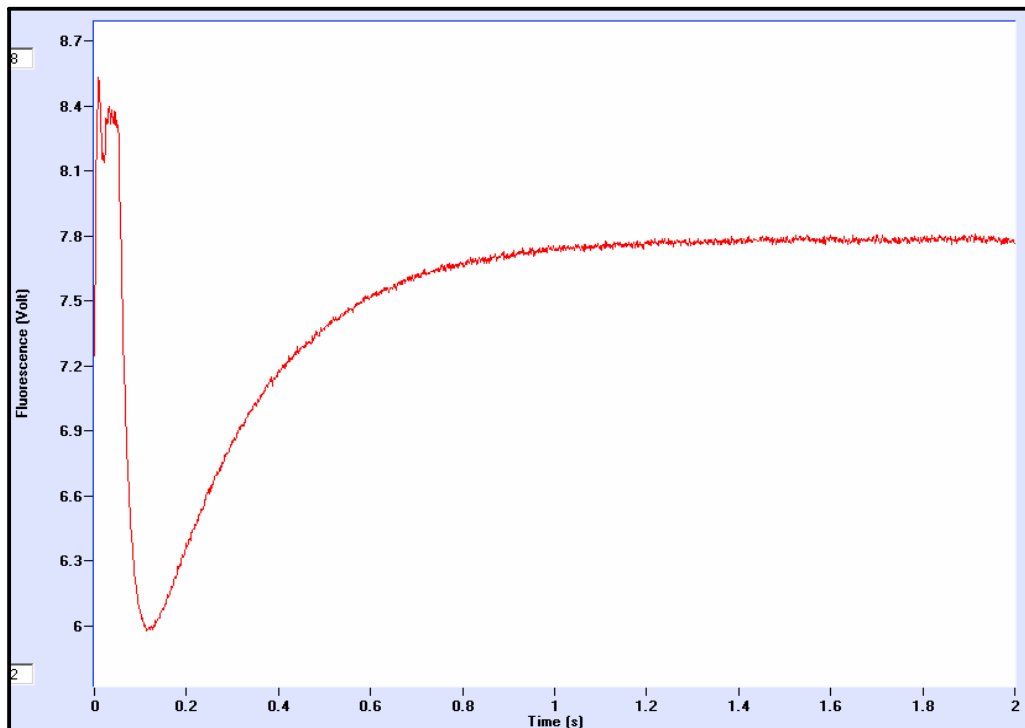

Figure 4 : lysosyme refolding followed by anisotropy

Figure 5: lysosyme refolding followed by fluorescence