Structural Genomics Consortium

Small Molecule Screening Workshop

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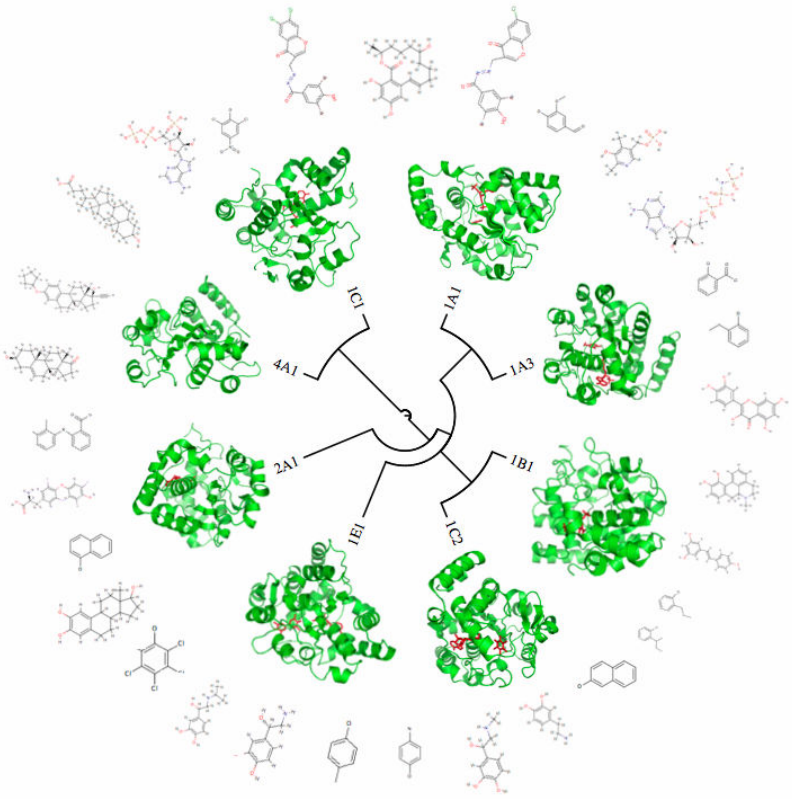
Toronto, Canada



Molecular Biophysics Group

How to prepare and run samples in the *FluoDia* T70

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Purpose:
Identify ligands for different proteins using a generic and high throughput method

Principle:
Equilibrium binding of a ligand increases the thermal stability of a protein in a manner proportional to the concentration and binding affinity of the ligand (Matulis et al, (2005) *Biochemistry* 44, 5258-5266; Vedadi et al, (2006) PNAS 103 (43), 15835-40)

Material needed:

1) 96-well plates
2) 384-well plates
   (Hard-Shell PCR plates: BioRad, catalogue number HSP3801)
3) Protein sample(s) at 10x the final concentration (e.g. 20 µM if the final concentration is 2 µM)
4) SYPRO orange
   (SYPRO orange: Invitrogen, catalogue number 56650)
5) HEPES screening buffer (100 mM HEPES, 150 mM NaCl, pH 7.5)
6) 96-deep-well plate (if needed) containing compounds at desired concentrations
7) mineral oil (Sigma, catalogue number M-1180)

Instrument:

FluoDia T70 is a filter-based high temperature fluorescence microplate reader manufactured by Photon Technology International (http://www.pti-nj.com/FluoDia_T70.htm).

Pre-screen protein concentration assay:

In order to determine if the protein is suitable for screening with the FluoDia and to select the protein concentration that will yield reproducible data you should perform an experiment (pre-screen) in which the protein is tested at three different concentrations: 2, 5 and 10 µM. It may be necessary for you to measure the protein concentration again (via A_{280} absorbance) to ensure the test is as accurate as possible.

1) Prepare a 275x stock solution of Sypro Orange (the stock solution is 5000x in DMSO) in the standard HEPES screening buffer.

2) For each protein concentration (2, 5 and 10 µM), prepare 55 µl of solution in the standard HEPES buffer and then add 1 µl of the 275x Sypro Orange solution to each (Sypro Orange is used at 5x final concentration).

3) Aliquot 25 µl from each test solution into separate wells of a 384-well FluoDia plate, layer 10 µl of oil on each well, spin the plate for one minute at 3000 RPM and run the temperature scan experiment.

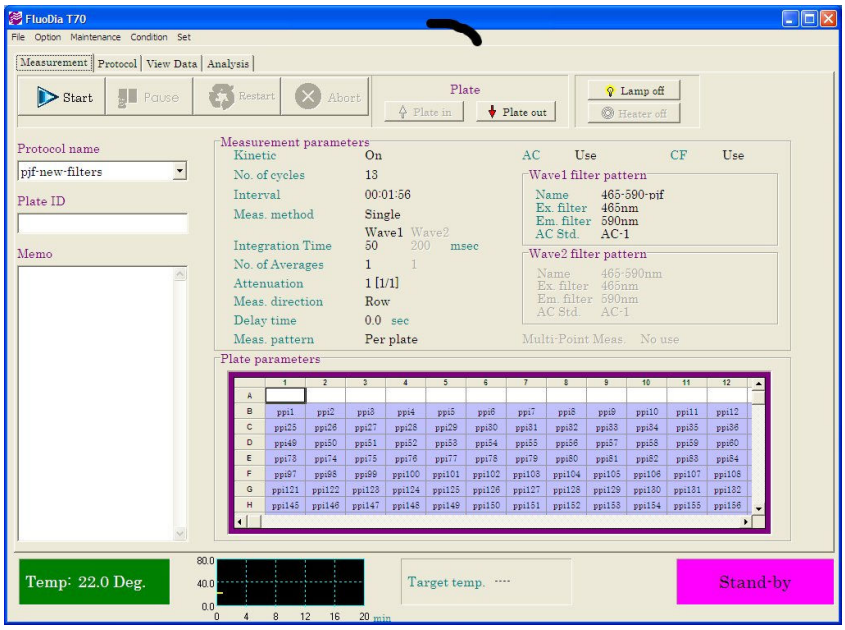
4) Using either the curves displayed by the FluoDia software or those generated after processing the data with BafFConv and BioActive (proprietary software), select an appropriate protein concentration to be used for screening purposes based on the reproducibility of the data measured for the duplicate wells. In some cases, the protein may have a high fluorescence background that makes it unsuitable for screening by FluoDia.

Sample preparation for screening:

1) If you are screening the protein at 2 µM (final concentration), prepare a stock solution consisting of 10x protein (20 µM) and 50x Sypro Orange. If the results of the pre-screen experiment (above) indicate that a higher protein concentration is required, prepare a solution containing 10x of that concentration of protein. In all cases the Sypro Orange dye should be at 50x concentration in the protein stock solution. Prepare a volume of this solution sufficient for adding 6 µl to each screening condition while also taking into account pipetting errors.
2) Prepare the compound solutions at desired concentrations. For 100 µM compound (final concentration), prepare 112 µM of each compound and transfer them into wells of a labeled 96-well plate.
3) Label the rows of another 96-well plate with the corresponding destination row in the 384-well plate as well as with the name of the protein you are characterizing.
4) Using a 12-channel pipette, aliquot 54 µl of each compound into the appropriate wells in the 96-well plate.
5) Using a 12-channel pipette, aliquot 6 µl of the protein / Sypro Orange solution (20 µM protein, 50x Sypro) into each well containing the compounds.
6) Using a 12-channel pipette, mix the solution, and then transfer 25 µl from each well in the 96-well plate into the corresponding destination well in the 384-well plate.
7) Layer 10 µl of mineral oil on each well in the 384-well plate using the 12-channel pipette
8) Spin the plate for 1 minute at 3000 RPM
9) Begin the FluoDia experiment

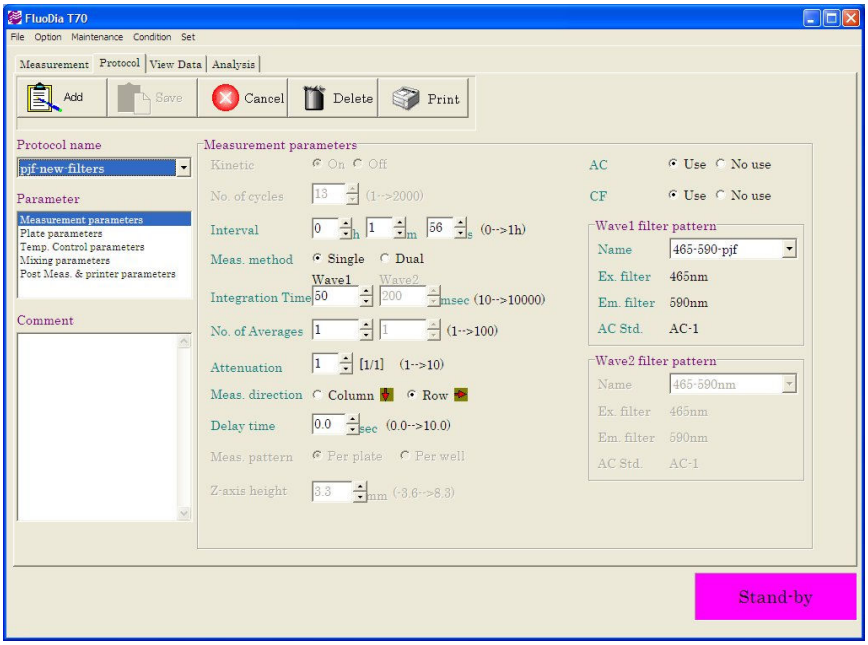
Running FluoDia:

1) Turn ON the power supply of the FluoDia instrument, start the *FluDia*T70 software, log in to the program using the appropriate username and password and let the system initialize.
2) After system initialization, the program's main screen will appear similar to the screenshot shown below:



This screen will show you the parameters of the previous experiment such as the set of filters used, the integration time, the number of cycles etc...

3) To create or open your own protocol click on the Protocol tab and the following window will appear:

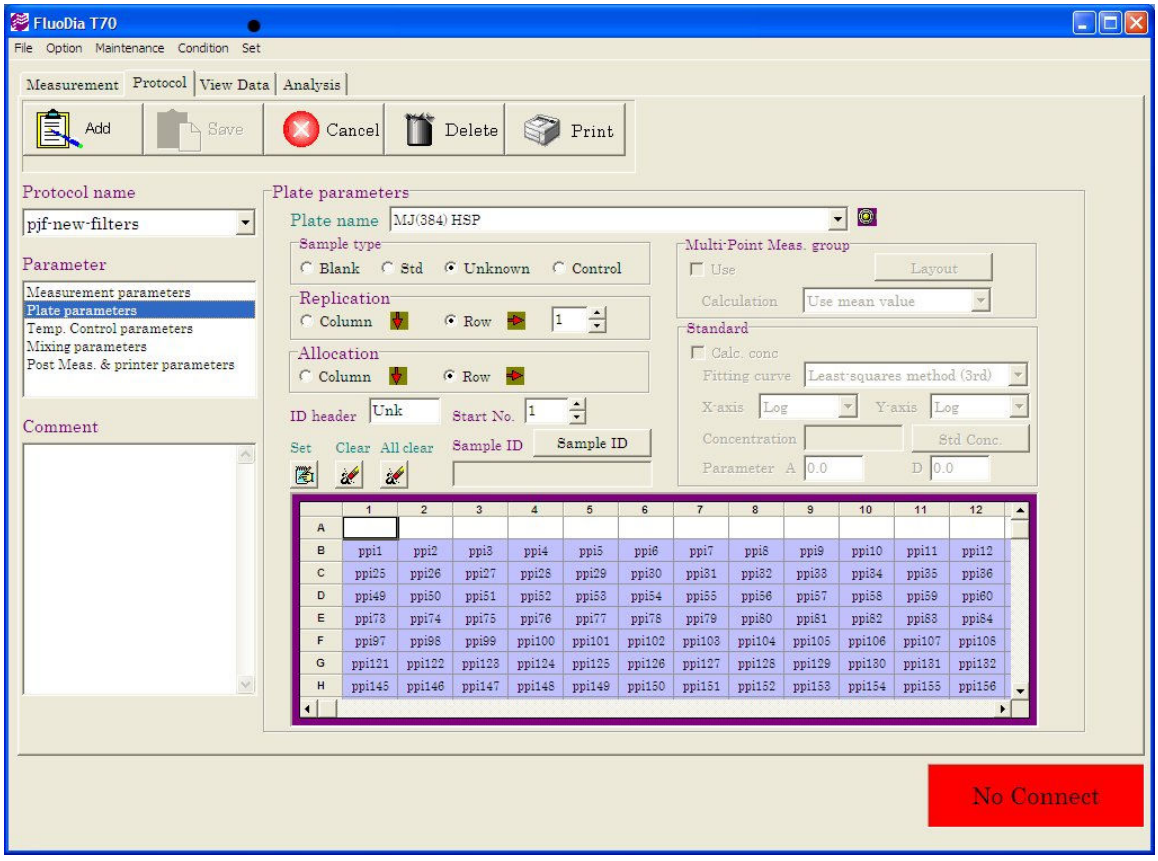


4) Use the following settings for the temperature scan experiment:

- Kinetic should be ON. Otherwise click the Plate parameter tab and select Temp. Stepping Mode and return to the protocol window
- Set measurement method to Single (one wavelength)
- Set interval to 123 sec (measurement time for a **full plate**). This will be the waiting time between the start of two continuous cycles.
- Set the integration time to 50 ms (exposure time for the PMT detector)
- Set number of averages to one, since we want the PMT to collect data once for each measurement
- Set attenuation to 1, because we want the measurement result = measured value (this can be changed if the measured data is too high)
- Select the measurement direction (row)
- Set delay time to 0.1 sec
- Make sure that you are using the right set of filters (for SYPRO orange use the filter set 465nm-590nm for excitation and emission wavelength, respectively).

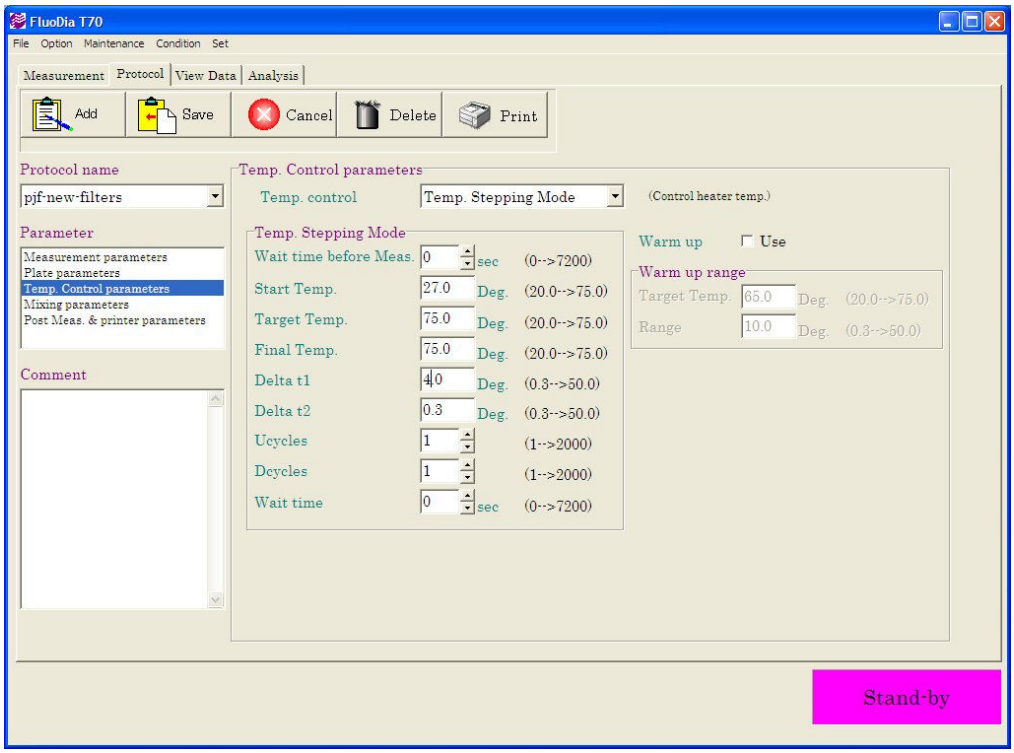
5) Use the **Add** button to give the new protocol a name and save it by clicking the **Save** button after you have completed editing it.

6) Click on Plate parameter item from the Parameter listing in order to enter sample information such as the name assigned to each well. All available positions in the microplate illustration should be filled in with a sample name (e.g. Unk). You may use any text you would like but there must be some present to ensure that all of the wells will be scanned since the number of wells selected for scanning affects the rate at which the FluoDia performs the temperature scan.



- Choose the plate type: MJ(384) HSP
- You can label the wells as Blank, Standard (Std), Unknown (Unk) or control. You can also put your own label by writing something in the ID header or use Sample ID if you want. When finished, click Set to have your label set in each selected well.
- If you process the data using BioActive (proprietary software), which plots and fits the data for all 384 wells in parallel, note that the resulting curves will be labeled according to the Excel map rather than with the names you provided in the FluoDia.

7) For temperature stepping setup, select the Temp. Control parameter item from the Parameter listing. A screen similar to that shown below will appear:



8) This is where you may enter the temperature control parameters. Use the following values:
   - Set Start Temp. to 27 °C
   - Set Target Temp. and Final Temp. to 75 °C
   - Set Delta t1 to 3 °C so that the temp increase occurs over about 1 hr.
   - The Delta t2 parameter can be ignored since we do not measure the temperature decrement between two steps.
   - Ucycles and DCycles are set to 1 because we are doing only one measurement per experiment.
   - Wait time is set to 0.

9) The FluoDia can mix the samples during the experiment if this option is configured in the Mixing parameters item from the Parameter listing. However, this option is not used for our routine screening.

After all of the parameters have been entered properly, save them and go back to the measurement window (by clicking the Measurement tab) to start your run. Once the temperature reaches the start of the experiment (27 °C), the first fluorescence measurement scan will start. When the scan is finished the system will heat the plate until the target temperature is reached. If we choose the Delta t1 to be 3 °C, the next target temperature will be 30 °C (27 + 3). When the final temperature of 75 °C is reached, the experiment is finished and the software will prompt you to save your data. You can save the data and analyze your data using FluoDia software. However, you may have to analyze a subset of data points (wells) each time.

Save your file as a csv file so that it will be compatible with the BafFConv conversion software (proprietary software). After you have converted the data with BafFConv you can analyze all 384 data points in parallel using the BioActive program (proprietary software). Converting, plotting and labeling the data for all 384 samples will take 5-10 minutes.

When the FluoDia has cooled down, exit the FluoDia software and turn off the instrument.

Ordering information:

- SYPRO orange: Invitrogen, catalogue number 56650
- Hard-Shell PCR plates: BioRad, catalogue number HSP3801