

Monolith NT.115pico: CMI Getting Started Guide to MicroScale Thermophoresis

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Introduction

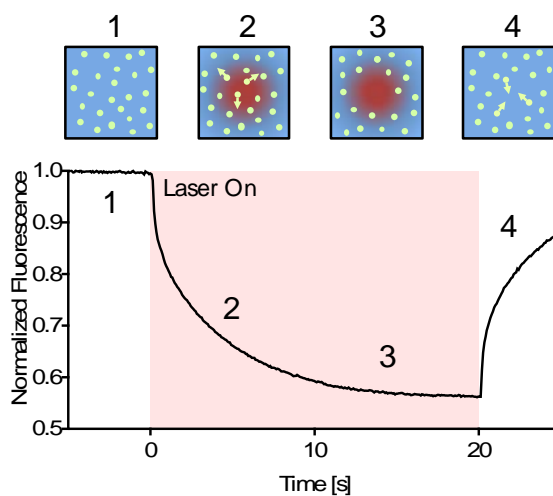
MicroScale Thermophoresis (MST) is an immobilization-free technology for measuring biomolecular interactions. The MST instrument detects the motion of fluorescent molecules along a microscopic temperature gradient, which reflects changes in the molecular hydration shell, charge, or size. Since one or all of these parameters will change with virtually every binding event, a wide range of biomolecules can be measured, from ions and small molecule fragments to large macromolecular complexes, in small volumes (~20 μ l), in a wide range of standard buffers and complex mixtures such as liposomes, detergent, serum, and cell lysates.

In an MST experiment, a local temperature difference (ΔT), induced by an infrared laser, leads to a local change in molecule concentration (depletion or enrichment), quantified by the Soret coefficient ST , $c_{hot}/c_{cold} = \exp(-ST\Delta T)$. The directed movement of molecules along a temperature gradient is termed "thermophoresis". Changes in the thermophoresis of a fixed concentration of a fluorescent molecule (the TARGET) in a constant buffer reflect changes in the size, charge, or solvation entropy of the fluorescent molecules due to binding of the non-fluorescent partner (the LIGAND). Measuring this behavior at different concentrations of ligand allows binding quantification and affinity determination.

Instrument Overview

The CMI has a **Monolith NT.115pico** from NanoTemper Technologies and two detectors:

- **Pico RED** detector:
 - Excitation wavelength: 600 - 650 nm
 - Fluorophore concentration ≥ 50 pM
 - KD range: pM-mM
 - Sample fluorophores: NT650, Alexa647, Cy5
- **Nano BLUE** detector:
 - Excitation wavelength 460-490 nm
 - Fluorophore concentration ≥ 5 nM
 - KD range: nM - mM
 - Sample fluorophores: fluorescein, GFP, AlexaFluor488



Key Features

- Fast measurement: K_D in about 10 min
- Wide K_D range from pM/nM-mM
- Low sample consumption: sample volume (<10 μ l per concentration)
- Immobilization free, in-solution measurements
- Measurements in complex mixtures (cell lysates, serum, detergents, liposomes)
- Wide size range for interactants (from ions to MDa complexes)

Required Supplies

- MST Capillaries
 - Monolith NT.115 Standard Treated Capillaries, MO-K002 (supplied by the CMI)
 - Monolith NT.115 MST Premium Coated Capillaries, MO-K025
- Labeling Kits (optional)
 - New 2nd Generation Labeling kits
 - Protein Labeling Kit RED-NHS 2nd Generation (Amine Reactive), MO-L011
 - Protein Labeling Kit RED-MALEIMIDE 2nd Generation (Cys Reactive). MO-L014
 - His-Tag Labeling Kit RED-tris-NTA 2nd Generation, MO-L018

Sample Preparation

Assay Buffers

- Many buffers are compatible with MST. It's usually a good idea to start with a buffer system in which your proteins are well behaved.
- **Addition of 0.05% Tween 20 or other detergent is usually required** to prevent sticking of proteins to the capillaries.
- Each capillary should be prepared with identically matched buffer.
- **Assay buffer** (with detergent) is used to dilute the fluorescent molecule to 2X.
- **Ligand buffer** is used to dilute the ligand and should match the highest concentration of ligand
- 0.5-1 mg/ml BSA can also be used to minimize non-specific binding.
- Buffer cannot be opaque.
- High viscosity samples may be hard to fill (up to 10% glycerol is fine).

Samples

- All MST experiments are setup with one fluorescently-labeled molecule (the **Target**) at a fixed concentration mixed with various concentrations of a non-fluorescent molecule (the **Ligand**).
- Concentration should be accurately measured
 - Errors in Target concentration can affect fluorescent signal and may affect the fit
 - Errors in the Ligand concentration will directly translate to errors in the K_D
- Protein aggregates will interfere with MST
 - Filter or centrifuge samples before use.
 - Assess protein heterogeneity via light scattering.
 - Purify protein samples with soluble aggregates by size-exclusion chromatography.

Target Sample (the fluorescent molecule).

- ~200 μ L/titration at >2X working concentration
- 5-20 μ M unlabeled protein, if using a chemical labeling kit
- RED detector:
 - Stock concentration of labeled Target: > 10 nM
 - Recommended working concentration: 5 nM (for $K_D > \text{nM}$)

- Minimal working concentration: ≥ 50 pM (used for K_D in pM range)
- BLUE detector:
 - Stock concentration of labeled Target: > 20 nM
 - Recommended working concentration: 20 nM
 - Minimal working concentration: ≥ 5 nM

Ligand Sample (the non-fluorescent binding partner)

- ~ 20 μ L/titration, at 2X working concentration (bring the highest stock concentration available for an unknown K_D)
- Recommended stock concentration $\geq 100X$ the expected K_D
- Recommended working concentration $\geq 50X$ K_D

Getting Started with MicroScale Thermophoresis using the Monolith NT.115pico

Resources

Additional resources are available at the instrument, including instrument and software manuals.

Experimental Design Tips

- When chemically labeling the target, aim for a labeling efficiency of 1 fluorophore per molecule (under-labeling is generally better than over-labeling).
- For high-affinity interactions, first optimize your assay with 5-10 nM labeled protein, then lower the concentration of fluorescent molecule to near the K_D (50 pM recommended lower limit).
- Prepare 20 μ l of sample to minimize pipetting errors and avoid problems associated with evaporation or sticking.
- Use the smallest tube (PCR tubes) when preparing samples to minimize adsorption.
- Spin down sample stocks for 5-10 min at 13000 RPM to remove large aggregates, which are a major source of noise.
- Use 5-90% LED power to excite the fluorophore.
- Target range for fluorescence is 3000-15000 units (25000 units is saturating).
- Use 20-60% laser power (low, medium or high MST power) to create a microscale temperature gradient of 2-6 C (default is medium power = 40%).
- Use a minimum of 12 titrant concentrations (16 is highly recommended).
- Replicate measurements are highly recommended.
- Replicates must use the same excitation power, MST power, temperature to be merged in the analysis software.

General Care and Maintenance

- Return the capillary to instrument after use.

Startup

1. Book time on the PPMS calendar before you start.
2. Login to the computer using your PPMS credentials (eCommons ID and password).
3. Turn on the Monolith NT.115pico (back of instrument, lower left).

4. Start the MO.control software.
5. Prepare stocks of target and ligand and buffers as described above.
 - a. Use MO.control software for instructions on preparing sample dilutions.

Data Collection

The default Monolith NT.115 control is MO.control. The MO.control software provides step-by-step instructions for sample preparation and includes data quality assessment tools for the Pretest, Binding Check, and Binding Affinity Experiment Types. The previous version of the control software (called NT.control) is still available for use

Basic Experimental Procedure (details on subsequent pages)

1. Start a New Session (or open a previous session).
2. Name the session and save it to your lab folder.
3. Select Fluorescence Channel (RED or BLUE).
4. Choose an Experiment Type:
 - a. **Pretest (highly recommended)**, tests your Target molecule for fluorescence intensity, adsorption on the capillaries, variation and sample aggregation (uses 2 capillaries)
 - b. **Binding Check (optional)**, tests the signal to noise by comparing MST signals from Target alone to Target + Ligand (uses 8 capillaries)
 - c. **Binding Affinity**, a standard MST binding experiment (uses 16 capillaries)
 - d. **Expert Mode (optional)**, allows more flexibility in the experimental setup. It is similar to setup in the old NT.control software.
5. Set Temperature Control (optional, but highly recommended).
 - a. Turn on Temperature Control (button near top right).
 - b. Choose temperature (22-45 C).
 - c. Click Set.
6. Analyze Data.
 - a. MO.Control Results for simple analysis at the time of data collection.
 - b. MO.Affinity Analysis software should be used for regular fitting.
7. Shut down Instrument.

Pretest Experiment (Recommended for all new Targets and/or buffers)

1. Plan Your Experiment.
 - a. Target
 - i) Name your Target (the fluorescent molecule); default "MyTarget."
 - ii) Enter the stock solution concentration for the fluorescent Target.
 - iii) Assay Concentration is auto-calculated based on the detector type (5 nM for pico RED and 20 nM for nano BLUE). You can change the concentration in the assay by clicking the pencil icon.
 - b. Describe your assay buffer.
 - c. Choose the capillary type.
 - d. System settings

- i) Enable auto-detect for Excitation Power, or
 - ii) Test the fluorescence manually and choose an excitation power such that fluorescence > 3,000 for the Pico RED detector and > 300 for the Nano BLUE detector.
 - iii) Use default MST power (Medium).
 - e. Temperature Control (optional, but highly recommended)
 - i) Turn on Temperature Control (button near top right).
 - ii) Choose temperature (22-45 °C).
 - iii) Click Set.
 - f. Click the "Go to Instructions" button (at the bottom right).
2. Instructions
 - a. Follow the onscreen instructions to prepare your samples.
 - b. Fill 2 capillaries with the same concentration of Target and load the capillary tray.
 - c. Insert the tray into the instrument.
 - d. Click the "Start Measurement" button (at the bottom right).
 3. Results
 - a. Examine the capillary scans (performed before and after MST for each of 2 capillaries) for fluorescence intensity, adsorption and variation.
 - b. Examine the MST trace for signs of aggregation.
 - c. Click the Review buttons for more details.
 4. A next step will be recommended at the bottom left of the Results (eg. Pretest of Binding Affinity).
 - a. Follow this link to setup your next experiment and save settings
 - b. Change the link as needed.

Binding Check Experiment (optional) or Binding Affinity Experiment

1. Plan Your Experiment.
 - a. Target
 - i) Name your Target (the fluorescent molecule); default "MyTarget".
 - ii) Enter the stock solution concentration for the fluorescent Target.
 - iii) Assay Concentration is auto-calculated based on the detector type (5 nM for pico RED and 20 nM for nano BLUE). You can change the concentration in the assay by clicking the pencil icon.
 - b. Ligand
 - i) Name your Ligand; default "MyLigand"
 - ii) Estimate the K_D (optional).
 - iii) Enter the concentration of your stock solution of non-fluorescent Ligand molecule.
 - iv) The highest concentration in this assay is auto-calculated as $\frac{1}{2}$ the stock concentration if no K_D estimate is made, or 50X K_D when a K_D estimate is made. You can also change the concentration in the assay by clicking the pencil icon.
 - v)
 - c. Describe your assay buffer.
 - d. Choose the capillary type.
 - e. System settings
 - i) Enable auto-detect for Excitation Power, or

- ii) Test the fluorescence manually and choose an excitation power such that fluorescence > 3,000 for the Pico RED detector and > 300 for the Nano BLUE detector.
 - iii) Use default MST power (Medium).
 - f. Temperature Control (optional, but highly recommended)
 - i) Turn on Temperature Control (button near top right).
 - ii) Choose temperature (22-45 °C).
 - iii) Click Set.
 - g. Click the "Go to Instructions" button (at the bottom right).
- 2. Instructions
 - a. Follow the on-screen instructions to prepare your samples.
 - b. Fill 16 capillaries and load the capillary tray (**put the high concentration in position 1**).
 - c. Insert the tray into the instrument.
 - d. Click the "Start Measurement" button (at the bottom right).
- 3. Results.
 - a. Examine the capillary scans for fluorescence intensity, adsorption, and variation. Examine the MST trace for signs of aggregation.
 - b. Examine the dose-response for signal to noise.
 - c. Click the Review buttons for more details.
 - d. Proceed to MO.Affinity Analysis Software for more complete data analysis and to merge data.

Expert Mode Experiment (optional)

- 1. Plan Your Experiment.
 - a. Select the first and last capillary to scan.
 - b. Select Excitation power (start at 20%).
 - c. Select MST power (Low = 20%, Medium = 40%, High = 60% power, use older NT.control software for other MST power settings).
 - d. Enter the final Target and Ligand concentrations (in nM) for each capillary or use "Add Dilution Series" to propagate the table with a defined dilution series.
- 2. Results
 - a. Examine the capillary scans for fluorescence intensity, adsorption, and variation. Examine the MST trace for signs of aggregation.
 - b. Examine the dose response for signal to noise
- 3. Open the MO.Affinity Analysis Software to fit the data (see below).

Data Analysis

- 1. Start the MO.Affinity Analysis software.
- 2. Load raw data from Home Tab (or from Data Selection Tab).
- 3. Data Selection Tab
 - a. Create New Analysis, choose Type:
 - i) MST for standard analysis.
 - ii) Initial Fluorescence, when fluorescence quenching or enhancement is observed (be sure to perform SD test to rule out adsorption).
 - b. Drag first experiment (or click + under experiment) to Analysis Set 1.

- c. Drag additional experiments onto Analysis set to merge replicates (merged data must be collected at the same MST power and Excitation power).
4. Dose Response Tab
 - a. Click Raw Data Inspection (top right) and examine.
 - i) Capillary Scans.
 - ii) Capillary Shape (all symmetrical and superimposed).
 - iii) Initial Fluorescence ($\pm 10\%$ of average).
 - iv) Bleaching Rate (minimal).
 - b. To ignore outliers during analysis, expand the dataset and click the capillary checkbox (to change to thumbs down).
 - c. Compare Evaluation Strategies.
 - i) Enter Expert Mode by clicking lightning bolt next to analysis set.
 - ii) Select the strategy best for your data (or use default on time).
 - d. Select K_D Model to fit data to a 1:1 binding model.
 - i) Enter fluorophore concentration in Target Concentration guess box.
 - ii) Fix the Target Concentration (optional).
 - e. Select Hill Model to include cooperativity (or if K_D model does not fit due to insufficient data).
 - f. Generate Full Report (top right) will create a PDF report of the data.
 - g. Curves can be exported as images or data using the export menu at the top of each curve.

Shutdown

1. Clean up in and around the instrument.
2. Discard capillaries in the sharps container.
3. **Return capillary tray to the instrument and close the door.**
4. Close the control and analysis software.
5. **Turn off the instrument power.**
6. **Logoff from PPMS!**

Book time and Report Problems through the PPMS system: <https://ppms.us/hms-cmi>

- rates are based on *booked and real-time usage*.

Contact cmi@hms.harvard.edu with questions.

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