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Use of Microscale Thermophoresis to Measure Protein-Lipid Interactions

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TITLE:

Use of Microscale Thermophoresis to Measure Protein-Lipid Interactions

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Phosphoinositide; Phosphatidylinositol 3-phosphate; FYVE domain; Vacuole; Lysosome; Endosome

SUMMARY:

Microscale thermophoresis obtains binding constants quickly at low material cost. Either labeled or label free microscale thermophoresis is commercially available; however, label free thermophoresis is not capable of the diversity of interaction measurements that can be performed using fluorescent labels. We provide a protocol for labeled thermophoresis measurements.

ABSTRACT:

The ability to determine the binding affinity of lipids to proteins is an essential part of understanding protein-lipid interactions in membrane trafficking, signal transduction and cytoskeletal remodeling. Classic tools for measuring such interactions include surface plasmon resonance (SPR) and isothermal calorimetry (ITC). While powerful tools, these approaches have setbacks. ITC requires large amounts of purified protein as well as lipids, which can be costly and difficult to produce. Furthermore, ITC as well as SPR are very time consuming, which could add significantly to the cost of performing these experiments. One way to bypass these restrictions is to use the relatively new technique of microscale thermophoresis (MST). MST is fast and cost effective using small amounts of sample to obtain a saturation curve for a given binding event. There currently are two types of MST systems available. One type of MST

requires labeling with a fluorophore in the blue or red spectrum. The second system relies on the intrinsic fluorescence of aromatic amino acids in the UV range. Both systems detect the movement of molecules in response to localized induction of heat from an infrared laser. Each approach has its advantages and disadvantages. Label-free MST can use untagged native proteins; however, many analytes, including pharmaceuticals, fluoresce in the UV range, which can interfere with determination of accurate K_D values. In comparison, labeled MST allows for a greater diversity of measurable pairwise interactions utilizing fluorescently labeled probes attached to ligands with measurable absorbances in the visible range as opposed to UV, limiting the potential for interfering signals from analytes.

INTRODUCTION:

Microscale thermophoresis is a relatively new technique in determining disassociation constants (K_D) as well as inhibition constants (IC_{50}) between biochemically relevant ligands. The leading commercial retailer for MST (e.g., NanoTemper) offers two popular MST technologies: 1) Label free MST requiring a fluorescent tag, and 2) labeled thermophoresis using the inherent fluorescence of proteins dependent on the amount of aromatic residues present in a given protein[1]. A disadvantage of label-free thermophoresis is that in most cases, it does not allow for the measurement of protein-protein interactions. However, it may be possible to engineer proteins without aromatic amino acids such as tryptophan for use in label free thermophoresis[2].

MST measures the movement of particles in response to the induction of microscopic temperature fields initiated by an infrared laser in currently available technologies[1]. MST can be used to measure protein-protein interactions, protein-lipid interactions, protein-small molecule, competition experiments, and even interactions between small-molecules so long as one can produce enough signal separation. Additionally, MST allows for the measurement of membrane-protein based interactions embedded in either liposomes or nanodiscs. Labeled thermophoresis takes advantage of the use of fluorescently labeled tags allowing for chemically controllable separation of signal between ligand and analyte. K_D values can be obtained using thermophoresis for interactions involving protein binding at low nanomolar concentrations, which in most cases is a much lower concentration of protein than what is required for isothermal calorimetry (ITC)[3]. Additionally, MST does not have strict buffering requirements as required for SPR[4] and labeled thermophoresis can even be used to measure binding constants of proteins of interest from non-fully purified protein solutions[5] with genetically inserted fluorescent tags[6]. A disadvantage of MST is that kinetic parameters cannot be obtained readily for MST as in SPR[2].

Thermophoresis measurements depend on the local temperature difference of a solution. This heat can be generated from an infrared laser. The MST device has a fluorescence detector coupled to an infrared (IR) beam and can pick up changes in fluorescence from local concentration changes of the fluorescent molecules at the point where the IR laser is targeted. The MST device utilizes an IR targeted laser coupled directly to a fluorescence detector focused at the same point in which the heat is generated in the solution. This allows for robust detection of changes in temperature corresponding to the depletion of molecules at the point

of heat generated by the IR laser (thermophoresis). Measured fluorescence generally decreases closer to the IR laser in response to temperature increases. The differences measured as a result can be due to multiple factors including charge, size, or solvation entropy. These differences are measured as changes in fluorescence in response to induction of heat or movement of molecules from hot to colder parts of the capillary (thermophoresis).

When loading a capillary with a given solution, it is important to leave air at either end of the capillary and not load the capillary completely full. The commercial capillary holds about 10 µL of solution. One can achieve accurate measurements with 5 µL of solution so long as the solution is manipulated to the center of the capillary, there are no air bubbles (potentially degas prior to loading capillary), and one is careful loading the rack to not jostle the solution from the center of the capillary, where the infrared laser is targeted. If the laser does not come in contact fully with solution, the result will most likely be one of three unusable outputs for that concentration: 1) no or low fluorescence detection, 2) higher fluorescence detection (potentially with jagged peak), or 3) fluorescence detection within other values from given titration, but with a jagged and unrounded peak.

For labeled thermophoresis it is optimal to have a fluorescence signal above 200 and below 2000 fluorescence units[7]. The MST device uses a range of LED intensities from 0 to 100, which can be selected to achieve a signal above 200 or below 2000. Alternatively, one can use different concentrations of the labeled ligand to modify the fluorescence signal to an optimal level. It is important to run a cap scan with a given MST measurement as a reference when analyzing data, as a poor cap scan can often result in a point that may later be determined to be an outlier. Each run should take approximately 30 min if measuring a single MST power with a cap scan. The commercial devices allow changes in MST power. In older software versions this could be set from 0 to 100; and in later versions one can select low, medium, or high MST. To achieve robust traces, a researcher may need to try each of these and decide which MST setting results in the most robust data for a given interaction.

PROTOCOL:

1. Preparation of materials

1.1. Prepare phosphate buffered saline (PBS): 137 mM NaCl, 2.5 mM KCl, 10 mM NaH_2PO_4, and 2 mM KH_2PO_4, pH 7.4[1].

1.2. Prepare NTA-Atto 647 N dye. Dilute stock NTA-Atto 647 N dye to 100 nM from a 100% DMSO solution into PBS without Tween.

1.3. Express FYVE-His – Protein as a fusion protein in *E. coli* and purify using Ni-NTA and size exclusion chromatography[8].

1.4. Titrate the analyte in PBS buffer. If the analyte is in a different buffer, labeled MST is not particularly sensitive to minor buffer differences from molecules such as DMSO, unless contents of analyte buffer interact with labeled protein.

2. Preparation of the MST device

2.1. Turn on the power switch on the back of the device.

2.2. Open the control software and ensure that the laptop is on and in connected status on the computer attached to the device.

2.3. Enter fluorescence and MST settings for this experiment. Set **MST Before** to **3 s**, **MST** on **30 s**, and **Fluorescence recovery (Fluo.)** after **1 s**. Before measures initial fluorescence and does not require long times; MST is the actual amount of time for equilibrium to be reached after heat induction.

2.4. **Table of Capillaries**: For each capillary tube, enter the name of the target (ligand), the name of the ligand (analyte), the concentration of the target, and the highest titration concentration and use autofill titration ratio. For example, enter 50 nM for the target concentration of FYVE domain, FYVE domain for target name, Di-C8 PI3P for ligand name, and the highest concentration of 25000 nM selecting 1:1 and dragging down to autofill slots 2-16.

2.5. Run a **Cap Scan** to select the appropriate LED (20% LED (preset)) and adjust between 200 and 2000 fluorescence units for labeled MST. **Cap Scans** should show uniform rounded bell-shaped peaks.

2.6. Select a range of MST powers and enter values for each to test for the most robust binding fit and hit **Start Cap Scan + Measurement**, scanning different values to determine best operating conditions for given interaction being tested.

2.7. Determine the MST power with a best fit using the analysis software and the preset for thermophoresis with Tjump. Analyze fits according to most fluorescence separation between lowest and highest concentration as compared to the MST power with best fit and select the MST power for replicate trials.

2.8. Determine whether photobleaching has occurred between first and second run by going to the analysis software and switching the analysis to expert mode. Next, select fluorescence instead of thermophoresis for analysis. Select expert mode and then photobleaching.

3. Preparation of samples for labeled MST

3.1. Bring the His_8 FYVE domain solution to a concentration of 200 nM in PBS without Tween.

3.2. Bring the NTA-Atto 647 dye to 100 nM in PBS without Tween.

3.3. Mix the FYVE domain and NTA-Atto 647 dye at a 1:1 volumetric ratio and allow to sit at room temperature covered from light for 30 min.

3.4. Centrifuge mixture of NTA-Atto 647 N dye and protein for 10 min in a dark room using a tabletop centrifuge at approximately 8,161 x *g*.

3.5. Store mixture at 4 °C after the experiment or on ice during the experiment for reuse within a few hours if needed in order to keep protein from denaturing.

3.6. Use the NT concentration finder to determine concentration range needed for titration.

3.7. Bring Di-C8 PI3P to appropriate maximum concentration in water.

3.8. Titrate the analyte using a 1:1 serial dilution in PBS buffer for 16 concentrations based on the previous step. Unlike SPR, thermophoresis is not as sensitive to buffer differences.

4. MST of samples

4.1. Turn on the device and the attached laptop.

4.2. Press the up arrow on the front of machine and slide the capillary rack out.

4.3. Load capillaries in the rack with the highest concentration at position 1.

4.4. In the control software, select the Red Channel corresponding to NTA-Atto 647 N.

4.5. Enter concentration, position, and name information for each capillary in the **Table of Capillaries**.

4.6. Run a capillary scan by hitting **Start Cap Scan** at 20% LED (preset) and adjust according between 200 and 2000 fluorescence units using either LED intensity settings or concentration of ligand (labeled protein).

4.7. Select a range of MST power.

4.8. Start **Cap Scan + MST Measurement**.

4.9. Analyze using the analysis software.

5. Analysis of MST data

NOTE: The analysis software provided by Nanotemper is proprietary and is performed using M.O. Affinity Analysis. There are different ways to measure binding affinities based on either fluorescence or thermophoresis. Newer versions of this software are preset to automatically evaluate data using thermophoresis and are preset to use Thermophoresis with Tjump taking advantage of both measurements. Alternatively, one can select either Tjump alone or Thermophoresis alone. Additionally, the analysis software allows estimated affinity measurements using initial fluorescence. These settings can be accessed in expert mode only.

5.1. Set the evaluation strategy in the analysis software to expert by clicking the box for the lightning bolt next to a data set in the Data Selection screen. The Analysis Software is preset to analyze to MST Analysis; however, a researcher can select Create New Analysis and select Initial Fluorescence Analysis in order to estimate binding affinities based on initial fluorescence. Expert mode is also available for initial fluorescence. In the analysis described below, thermophoresis and Tjump was used to determine the K_D of the presented of FYVE domain with its natural substrate, the lipid phosphatidylinositol-3-phosphate (PI3P).

REPRESENTATIVE RESULTS:

This is a sample output using the affinity analysis. The labeled MST was used to determine the binding constant of the FYVE domain from Hrs to the soluble dioctanoyl (DiC8) PI3P of one of its natural substrates[9–11]. **Figure 1** presents the thermophoretic traces from one trial of a 1:1 titration of DiC8 PI3P starting at 25,000 nM against 50 nM of Cy5 labeled FYVE domain[12]. Initial fluorescence (time before infrared laser turned on), Tjump (time initially after infrared laser turned on), and thermophoresis (once particles reach equilibrium with temperature) are shown. One can calculate a K_D from any one of these measurements alone or can use a combination of Tjump and thermophoresis taking into account two of these measurements.

In **Figure 2**, a saturation curve is shown for the thermophoresis with Tjump output from the analysis software. As shown in **Figure 2**, saturation curve can be plotted from these results; however, it may be difficult to calculate a saturation curve as Fnorm does not start from zero. In order to get around this issue, the data can be manually normalized, or the output can be set to the fraction-bound determined by the analysis software. Generally, MST results are determined using a log-scale as shown in **Figure 3**. The analysis software automatically takes into account protein concentration in order to determine binding affinity by selecting the K_D model and inputting the protein concentration. The Hill model in the analysis software can also be used, which does not take into account protein concentration, but can potentially give a measure of cooperativity for a given interaction. Exporting either output from the analysis software and plotting in third party software, one can obtain a K_D measurement as shown in **Figure 3**. It should be noted that the critical micelle concentration (CMC) of dioctanoyl phosphoinositides is >3 mM, indicating that these experiments are operating far below the CMC[13].

FIGURE LEGENDS

Figure 1: MST traces of FYVE domain to DiC8 PI3P. Traces are shown for PI3P analyte to cy5 labeled FYVE domain ligand measured in the red channel. Initial fluorescence was measured at

room temperature for 5 s (**A**) prior to turning on infrared laser. Tjump (**B**) is measured in the initial seconds following induction of heat from the built-in infrared laser and measures fluorescence differences present when IR laser is first turned on prior to thermophoretic movement of particles being measured. Thermophoresis (**C**) is measured at a later time after molecules equilibrate from the movement induced by heat from the infrared laser due to thermophoretic motion differences due to factors such as size, charge, solvation entropy, or conformational change of particle being measured with respect to titrated analyte.

Figure 2: Saturation binding determined from exported MST results using third party software. Normalized fluorescence results exported from the analysis software. The data were exported and plotted using one-site specific binding model.

Figure 3: Binding affinity of FYVE domain to DiC8 PI3P using a sigmoidal model. Analysis of exported data from **Figure 1** exported from the analysis software using the normalization Fnorm [%]. Taking the log of the concentrations of DiC8 PI3P plotted against Fnorm using Graphpad Prism v.7 using Sigmoidal, 4PL, X is log(concentration) model allows a fit that results in a K_D of 890 ± 170 nM for FYVE domain binding affinity to DiC8 PI3P.

DISCUSSION:

The determination of FYVE binding to DiC8-PI3P provided a robust fitted K_D of 887 ± 169 nM for the given interaction, which is slightly lower affinity than the measured K_D of FYVE to PI3P liposomes, which was around 50 nM[9–11]. This difference is most likely due to the lack of a membrane, which generally results in lower affinity for membrane specific lipid binding interactions and therefore demonstrates the role for the liposome membrane scaffold to this interaction[9,14].

In order to further determine the strength of MST data, a researcher needs to examine both the shape of the traces from **Figure 1** and the separation of the traces from the chosen analysis method. Looking at the traces from **Figure 1**, most concentrations resulted in a clear trace as determined by the leveling off of the thermophoresis portion of the curve. However, for traces corresponding to 12,500 and 625 nM, the end part of the fluorescent measurement tailed upward at around 20 s. This could be due to aggregation in the sample adherence to the sample capillaries, which can be remedied by additions of either or both Pluronic or Tween to the buffer[15]. However, detergents such as these may not be suitable for protein-lipid interactions[16]. To test for non-specific binding for this type of protein-lipid interaction one can add in BSA, increase salt concentration, or use glycerol in the buffer and test whether this affects the estimated K_D[17,18].

Separation is determined by the difference in Fnorm between the highest and lowest concentration measurement for the given interaction. As shown in **Figure 2**, the separation for this interaction was approximately 75 fluorescence units. Generally, speaking, a separation of at least 5 fluorescence units should be achieved to confidently rely on MST data of unknown chemical affinity measurements. If a robust K_D fit is achieved at slightly lower separation, one

might be able to still consider such data if it corresponded to affinity measurements used via another technique such as SPR or ITC.

Because some of the thermophoretic traces showed some stickiness in the sample, the output chosen for **Figure 3** was a combination of thermophoresis with Tjump. This setting is the preselected setting in most later versions of the analysis software. **Figure 3** indicates a robust fit to a sigmoidal binding affinity model as there was clear saturation with a 12 point 1:1 titration. The device allow for 16 points to be taken in a given run, and many interactions require all of these points to achieve a strong binding affinity measurement. In order to achieve greater confidence, this trial should be repeated with new capillaries two or more times requiring a total of 36 capillary tubes for this experiment. Additionally, one might try another technique such as SPR to corroborate this data in order to ensure reliable reporting of affinity constants as we previously have done[8].

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DISCLOSURES:
The authors declare no potential conflict of interest.

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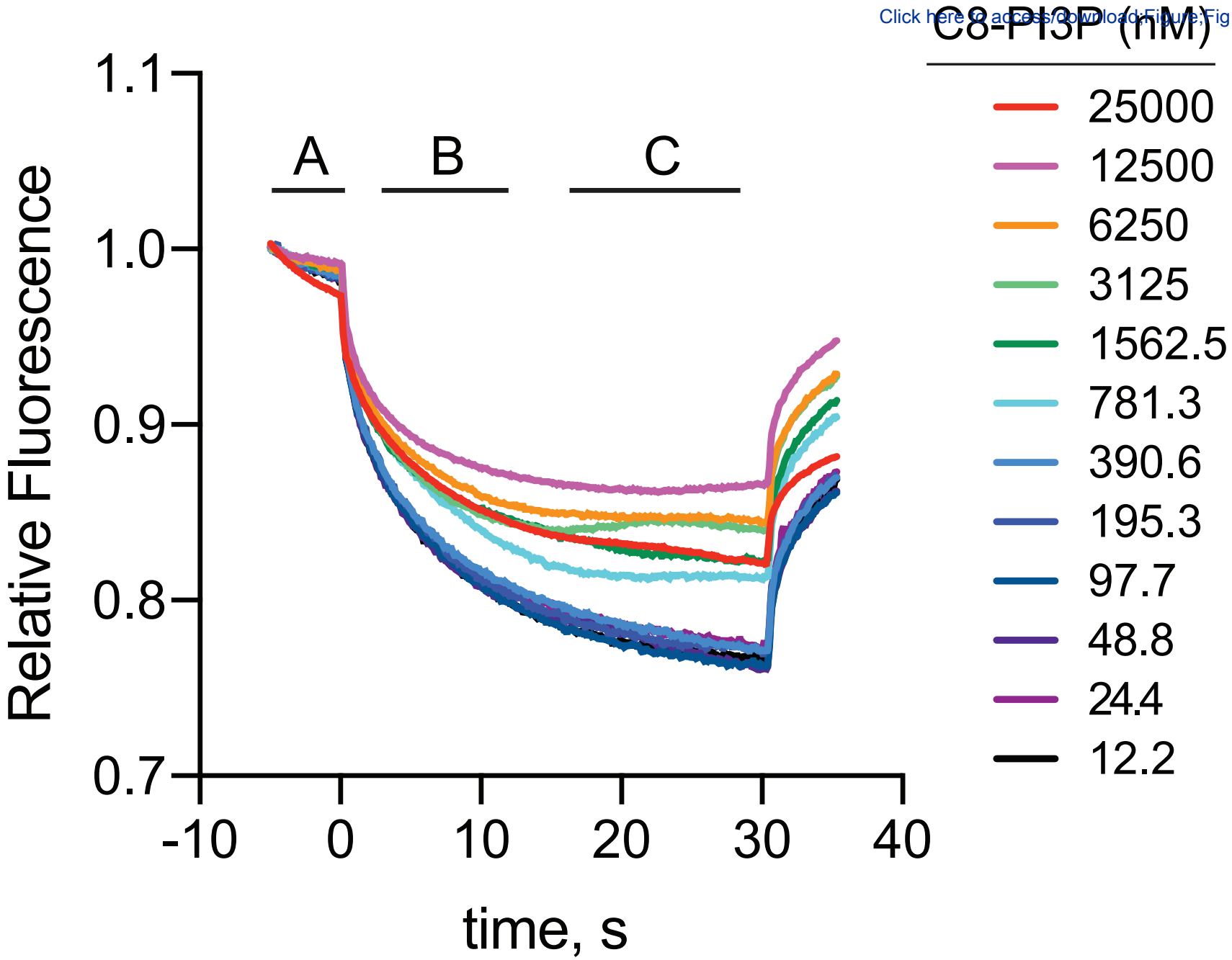
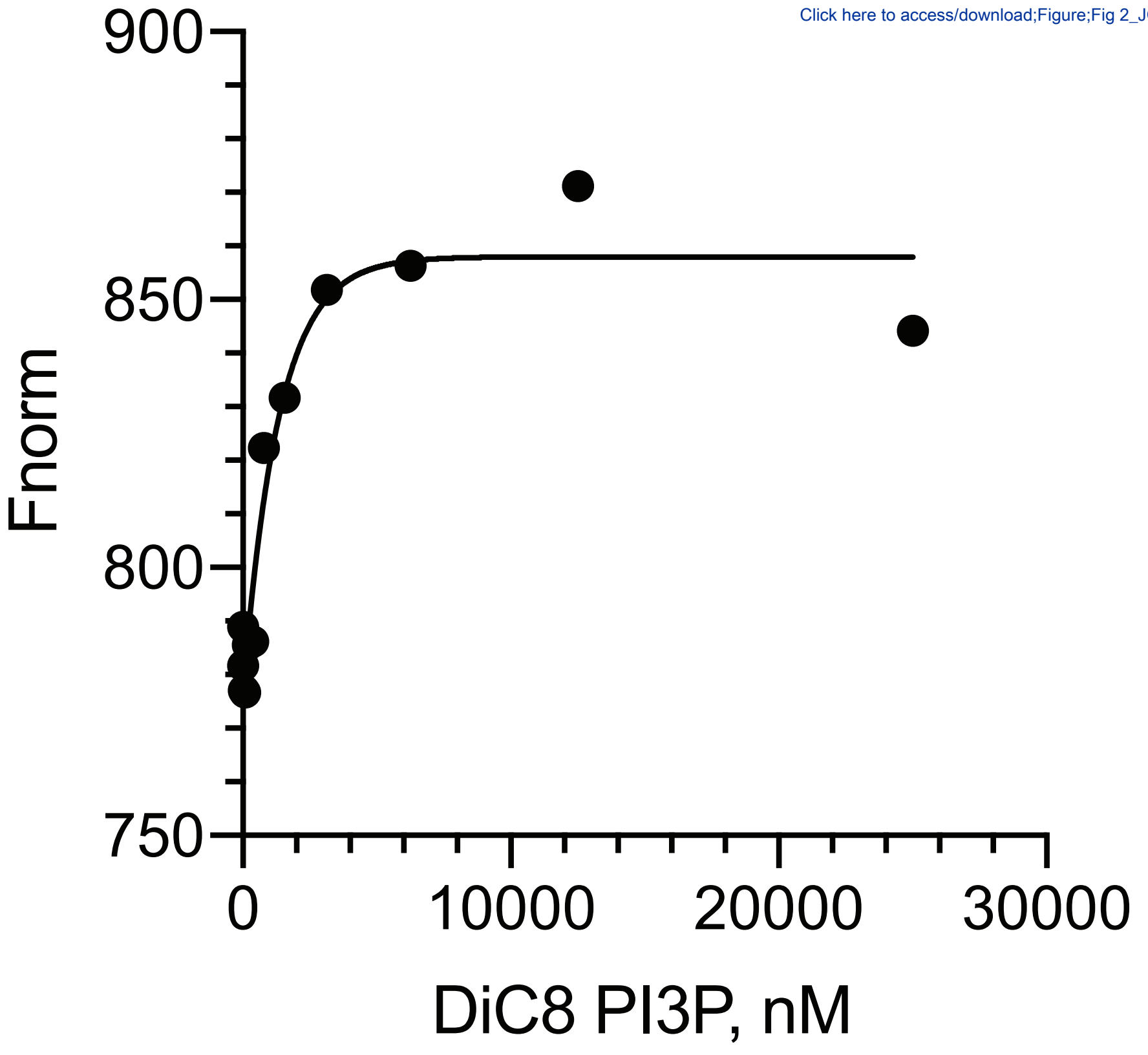


Figure 1

Figure 2



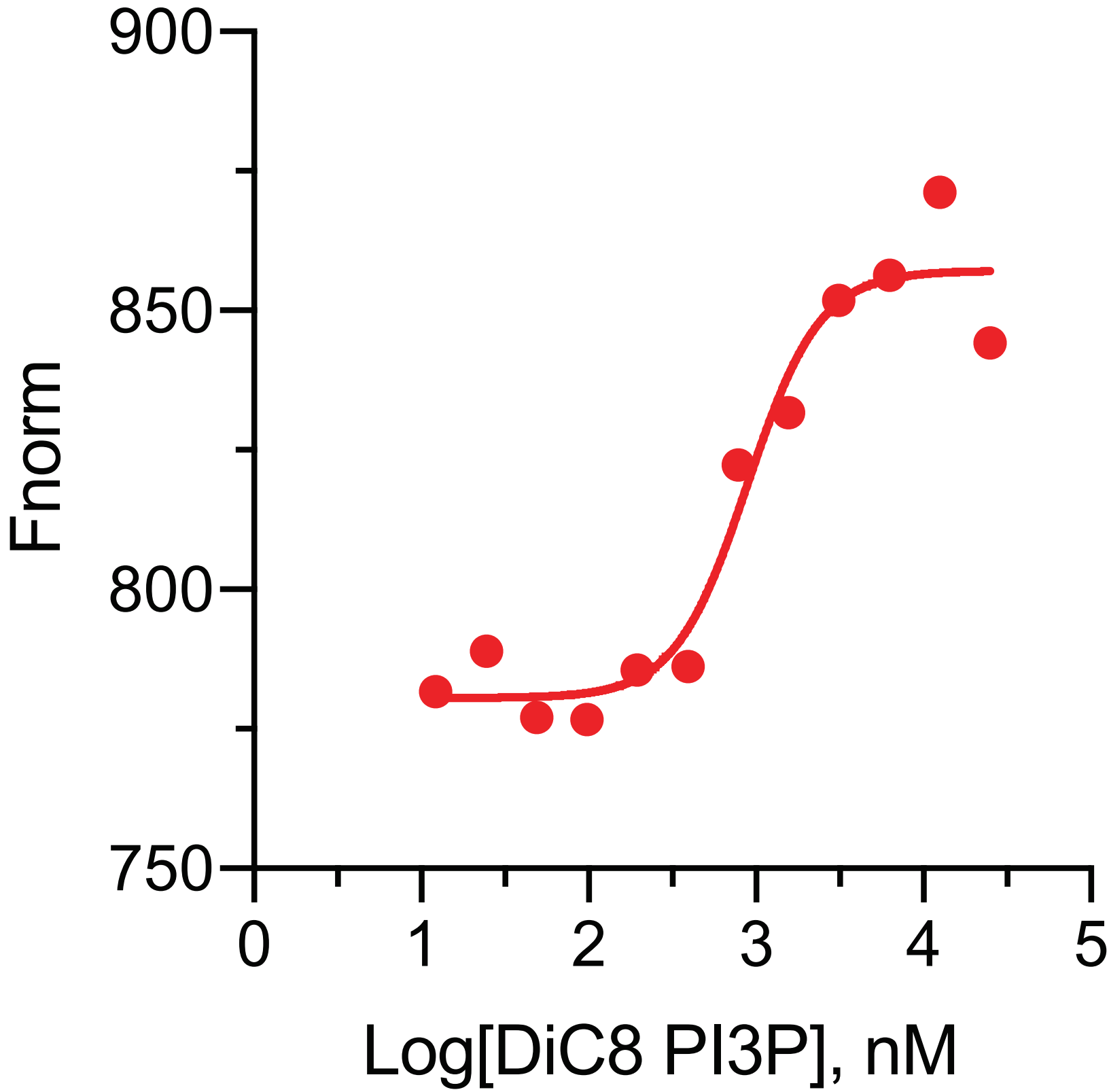


Figure 3

Table of Materials

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Name of Material/Equipment	Company	Catalog Number	Comments/Description
Cy5 Maleimide Mono-Reactive Dye	GE Healthcare	PA23031	For protein labeleing
Graphpad Prsim	Graphpad software		
Monolith NT.115 Capillaries (1000	Nanotemper	MO-K022	Capillaries for MST
Monolith NT.115 machine	Nanotemper		University equipment
NTA-Atto 647 N	Sigma	2175	label for His tags
Phosphatidylinositol 3-phosphate c	Echelon	P-3008	Lipid for binding experiments

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RE: JoVE60607

Title: Use of Microscale Thermophoresis to Measure Protein-Lipid Interactions

September 11, 2019

Dear Dr. Steindel

We have carefully addressed each of the reviewers' comments point-by-point. Our responses are listed after each of the reviewers' comments. Thank you for kindly considering this work.

Sincerely,
Rutilio A. Fratti

Editorial comments:
General:
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

2. Please revise lines 93-95, 129-134, 143-148 to avoid textual overlap with previous publications.

These have been revised

3. Please ensure that the manuscript is formatted according to JoVE guidelines–letter (8.5" x 11") page size, 1-inch margins, 12 pt Calibri font throughout, all text aligned to the left margin, single spacing within paragraphs, and spaces between all paragraphs and protocol steps/substeps.

This is now correct

4. Please do not include footnotes.

The footnote has been removed

5. Please remove the embedded figures from the text.

The embedded figures have been removed

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For example: Nanotemper Monolith, NT.Control, M.O.Affinity Analysis, etc.

Definitions have been added and generic language was used thereafter in the methods

Protocol:
1. Please give centrifuge speeds in terms of 'x g' instead of 'RPM'.

This has been converted to 8161 x g

2. For each protocol step/substep, please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. If revisions cause a step to have more than 2-3 actions and 4 sentences per step, please split into separate steps or substeps.

This has been addressed when applicable

Specific Protocol steps:
1. 2.3: It isn't clear what 'Fluo' is supposed to be.

Fluo. (Fluorescence recovery)

2. 5: Please include more detail about these analysis steps (e.g., specific menu options) if they are to be filmed.

More details have been added as suggested

References:
1. Please do not abbreviate journal titles.

The journal titles are now full length

Table of Materials:
1. Please ensure the Table of Materials has information on all materials and equipment used, especially those mentioned in the Protocol.

The table is complete

Reviewers' comments:
Reviewer #1:
Minor Concerns:
1) The end of the abstract distracts a little bit from the theme of the article. The main theme and protocol is measuring lipid-protein interactions but the end of the abstract goes into determine IC50 and titrating in different ligands. This is a little confusing and tangential.

The abstract has been modified to be less confusing

2) page 3 line 90-91 needs to rewritten

These lines have been rewritten

3) page 3 line 96-98 the authors may want to clarify use of resources (based upon protein abundance, etc. as each purified protein would be expected to be different).

This is now clarified

4) page 4 line 150: store on at 4C? Needs clarification.

Fluo. (Fluorescence recovery)

5) page 5 lines 179-182: run on sentence. Needs to be rewritten

This has been rewritten

6) page 8 line 248: the error has two significant figures. Should likely be 887 +/- 170 or 890 +/- 170

The significant figures have been changed as suggested

7) page 8 line 266: these types of reagents needs clarification? What type of reagents are the authors referring to in this sentence?

We now clarify that pluronic and tween were used

8) For the discussion: are there any concerns of nonspecific binding signal in this assay for lipid-protein interactions?

We now discuss that non-specific binding can be reduce by adding BSA or increasing salt concentrations.

9) The authors mainly have monitored protein-lipid interactions with soluble lipid, presumably below the CMC of the lipid. Are there any references or additions to discussion that can be added for measuring vesicle binding compared to soluble lipid?

The CMC value for diC8 phosphoinositides is >3mM. Thus, the concentrations used here are well below the CMC. We now include a reference that describes the partitioning of short chain phosphoinositides (Collins and Gordon, 2013, Biophysical Journal 105:2485-2494).

10) In the discussion, the authors refer to a manuscript by Gillooly et al. that determined the FYVE domain membrane affinity at ~50 nM. They may also want to cite work that determined similar affinity of the HRS FYVE for PI3P containing membranes (Stahelin et al. 2002 J. Biol. Chem., Blatner et al. 2004 J. Biol. Chem.) as well as the fact that these published works support the mechanisms the authors propose in lines 255-257 (that membrane affinity for PI3P is tighter than just the PI3P headgroup binding).

These references have been added.

Reviewer #2:

Major Concerns:

I have two major concerns with this manuscript.

1) The procedures described are overall complete standard and familiar to everyone who uses MST. The level does not go beyond anything that a first time user will be taught by the instrument administrator during the introduction. So it is really hard to see the additional value provided by this article, compared to the instrument manual and various application notes.

Also, the complexity of the tasks involved is such that they do not warrant, in my view, the production of a video. But I leave this decision to the editor.

We understand the reviewer's skepticism about the merits of a video. That said, we are constantly asked to show people how to do it, even though the instructions are in the manual. Unfortunately, analyzing the T-jump + Thermophoresis uses a proprietary algorithm, which is not available in the standard equipment set up. However, we can show the difference between the thermophoresis, T-jump and T-jump + thermophoresis in the video. We can also show the difference in binding of FYVE to PI3P short chain v. liposomes in the video. Moreover, it would be difficult and space consuming to show the output of all 3 as thermophoresis and T-jump can be changed manually to different time points, which can be easily shown in a video, but extraordinarily difficult to do in a paper. Thus, we chose to use the preset of T-jump + thermophoresis for the paper.

2) The preparation of the samples needs to be described in more detail. In particular, absolutely nothing at all is said about the preparation of the lipid sample. In what form are the lipid molecules present? What is the solubility of this lipid in water? I can't imagine that the lipid will be present as a molecular solution at the highest concentrations employed. How is it dissolved etc? A study that would present additional value for the reader would for example involve the comparison of the binding to liposomes and to lipids with are in a non-membrane form, as here.

This is now included on lines 169-170

Minor Concerns:
The binding curves should be shown and analysed with the various different ways of extracting the data from the MST curves, i.e. T-jump, thermophoresis and T-jump+thermophoresis. It should be discussed in what way they differ and which is the recommended way.

In Figure 1, T-jump, Thermophoresis, and Initial Fluorescence are indicated. We describe the differences in the text and in the Figure Legends. T-jump + Thermophoresis is what we used for Figure 2 and Figure 3 and we show how to transform the data using a sigmoidal binding curve. Unfortunately, analyzing the T-jump + Thermophoresis uses a proprietary algorithm, which is not available in the standard equipment set up. However, we can show the difference between the thermophoresis, T-jump and T-jump + thermophoresis in the video. We can also show the difference in binding of FYVE to

PI3P short chain v. liposomes in the video. Moreover, it would be difficult and space consuming to show the output of all 3 as thermophoresis and T-jump can be changed manually to different time points, which can be easily shown in a video, but extraordinarily difficult to do in a paper. Thus, we chose to use the preset of T-jump + thermophoresis for the paper.

Also the manuscript has poor language in places and needs some editing.

The manuscript has been carefully edited to remove poor language