

# Protein-Protein Interaction Analysis

Application Note NT-MO-013

## MicroScale Thermophoresis Measurements on *in vitro* Synthesized Proteins

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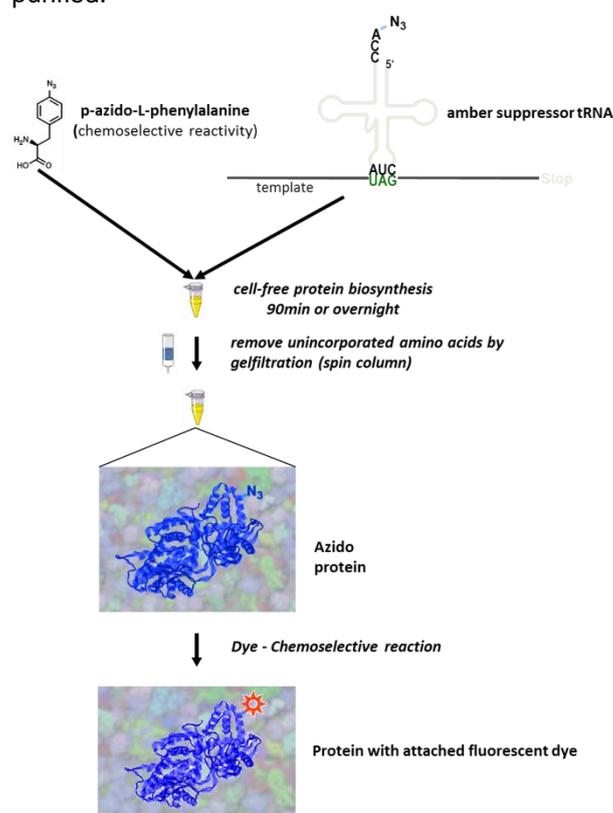
### Abstract

Nowadays, especially through the advent of systems biology, and the ability to generate predictive models, it is even more important to transform biology from a qualitative to a quantitative science. Interactions between proteins not only need to be identified, but their equilibrium rate constants need to be determined as well. Here we describe a very elegant and simple system to obtain quantitative data for protein-protein interactions using cell-free protein biosynthesis in combination with MicroScale Thermophoresis. We have been able to characterize the interaction of Calmodulin with  $Ca^{2+}$  as well as its ligand M13 straight in the *in vitro* synthesis reaction. Furthermore, we are demonstrating the generic applicability of this approach with another set of proteins, an Antibody fragment and Cyclophilin A. All this work has been done without tedious expression and prior purification of the interacting proteins.

### Introduction

It is crucial in Biology these days to provide not only qualitative data, but also quantitative data. Now, demonstrating an interaction is fairly simple and routinely done by high throughput methods such as yeast-two-hybrid or pull down assays, and MS. However, transforming this knowledge into quantitative data to feed into predictive models of regulatory networks of the cell is far from easy. Usually the process starts with the careful selection of the two interacting proteins, the right iso- or spliceforms and the selection of the appropriate domains. These have to be passed through construct design, as a lot of mammalian

full length proteins are not readily expressed in a heterologous system. Expressing these proteins is as well not easy and requires at least a laboratory with S1 biohazard safety standards. After being able to express the protein it still needs to be purified.



**Fig. 1** *In vitro* expression and labeling scheme! Protein samples can be used immediately after without purification for the synthesis mix.

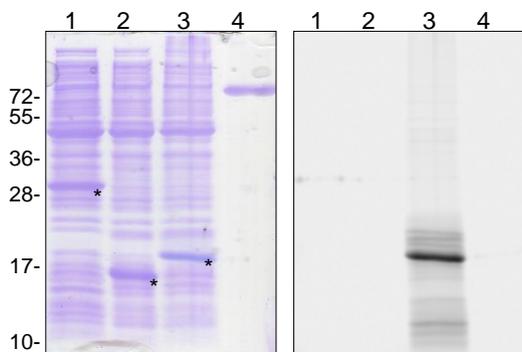
Purification of biomolecules for biophysical analysis requires high quality samples and a high degree of purification which is rarely achieved by a single affinity purification step. If one looks now at the long time standard methods like SPR, ITC or

NMR they all have their pitfalls. SPR requires a highly pure sample and a successful and time consuming immobilization strategy. ITC requires vast amounts of material that can be a challenge to produce at sufficient high quality.

The present study was designed to show that the novel and powerful technology MST used in combination with cell-free protein biosynthesis is a very efficient and smart shortcut to this classical approach to obtain quantitative protein interaction data. We have selected two interactions to demonstrate this possibility. Calmodulin and its interaction with an M13 peptide fused to citrine was analyzed in a first set of experiments, and as a second model system we used Cyclophilin A and a single chain antibody fragment directed against it.

## Results

Calmodulin was synthesized with a short leader sequence containing an amber codon, which allowed the site-directed introduction of the chemoselective reactive unnatural amino acid p-azido-Phenylalanine by expression in RiNAs RF1-depleted cell-free orthogonal protein translation system.

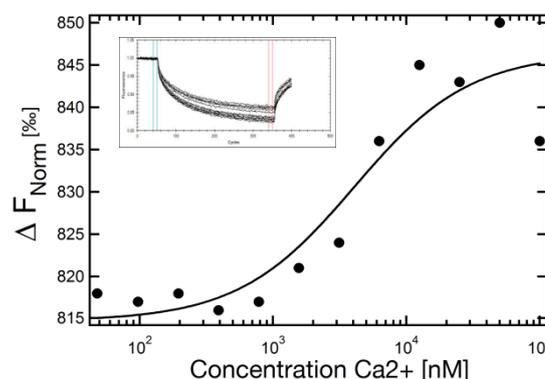


**Fig. 2** SDS-PAGE analysis of *in vitro* synthesized and dye-labelled Calmodulin and its interaction partner M13-Citrine. Left panel: Coomassie stained gel; right panel: Fluorescence Image (excitation 633 nm). Lane 1: M13-Citrine, lane 2: CaM, lane 3: CaM of lane 2 modified with DyLight650, lane 4: 0.75  $\mu$ g BSA as a standard. 1.5  $\mu$ l reaction each were loaded in lanes 1, 2 and 3. Positions of synthesized proteins are marked with asterisks (\*). Full conversion of synthesized CaM with dye is indicated by shifting to higher molecular weight (compare lane 2 and lane 3).

This amino acid was then selectively reacted with a fluorescent dye by means of Staudinger ligation, providing the labeled molecule for the MST analysis. The unlabeled interaction partner M13-Citrine (M13 peptide fused to Citrine) was expressed similarly.

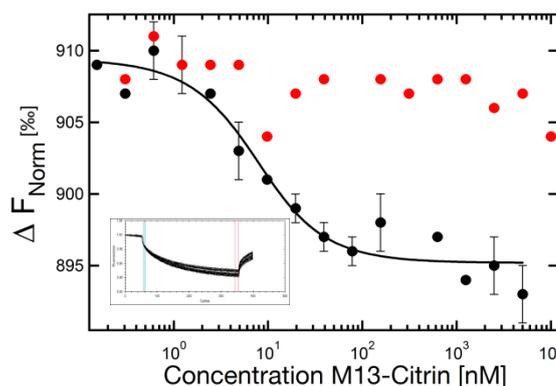
In order to first assess the quality of the *in vitro* synthesized Calmodulin we tested its binding to

Ca<sup>2+</sup> ions. Previously in a purified system we had shown an affinity of 2.8  $\mu$ M, and in this set of experiment we determined an affinity of 4.14  $\mu$ M.



**Fig. 3** Binding of Ca<sup>2+</sup> to Calmodulin. The  $K_d$  was determined to be  $4.14 \pm 2.26 \mu$ M. Inset displays the original normalized MST traces.

In a second step we titrated the synthesis reaction of cell-free synthesized M13 peptide as a fusion protein with citrine, named M13-Citrine, without any purification step to Calmodulin, and performed a further MST measurement. The affinity was determined to be 5 nM. This corresponds nicely with the literature value of 1 nM for the free M13 peptide (Blumenthal et al. 1985).

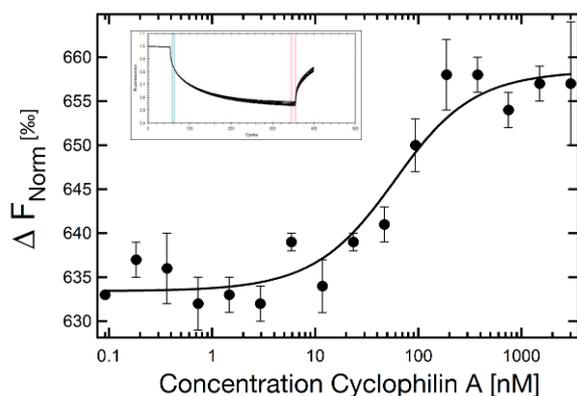


**Fig. 4** Binding of M13-Citrine to Calmodulin (black dots). The affinity was determined to a  $K_d = 4.48 \pm 1.77$  nM. As a control a non-binding mutant of M13-Citrine was used (red dots). No binding was observed. Inset displays the original normalized MST traces of the experiment using Calmodulin and M13-Citrine.

In a control experiment the synthesis reaction of a non-binding M13-Citrin mutant was used. An identical titration series has been prepared and analyzed in the same way as the M13 (wt)-Citrine interaction by MST. The corresponding analysis of the experiment showed no binding at all, as it was expected.

As a second interaction we investigated the one between Cyclophilin A and a single chain antibody fragment named AntiEC5218 directed against it.

Again the fluorescent dye was introduced within a leader sequence at the N-terminus of the antibody fragment, through a similar process as used for Calmodulin. A dilution series of an unlabeled Cyclophilin A *in vitro* synthesis reaction was prepared and mixed with the labeled AntiEC5218. These samples were loaded in MST standard treated capillaries and mounted in the NanoTemper Monolith. Analysis of the experiment yielded an affinity of 46 nM.



**Fig. 6** Binding of Cyclophilin A to AntiEC5218. The  $K_d = 46.0 \pm 17$  nM was derived through curve fitting. Inset displays the original normalized MST traces.

## Conclusion

In this study it was shown that quantitative binding data can be generated in a matter of a few hours using RiNAs *in vitro* translation systems for the synthesis of fluorescently labeled proteins as well of their unlabelled interaction partners in combination with NanoTemper's MicroScale Thermophoresis technology for interaction analysis. Non-purified proteins in whole synthesis reactions can be used after a short desalting step, at which volumes and protein concentrations of the cell-free protein biosynthesis reactions fit the needs of MST very well. Neither elaborate protein purification nor cell culture facilities or expensive HPLC and FPLC equipment are required to prepare interaction partners for the measurement of affinities.

Furthermore, the time to obtain these data is minimal. *In vitro* synthesis reactions take on average a few hours, at most overnight, while the MST experiment itself can be performed in a matter of minutes. The combination of both, allows the synthesis of the desired target proteins and the characterization of their interaction in less than a day.

## Material and Methods

### Protein Synthesis and Labeling

Protein synthesis was performed using an *E. coli* derived cell-free translation system depleted from termination factor RF1 (Gerrits et al. 2007, Serwa et al. 2009) and supplemented with enriched fractions of orthogonal amber suppressor tRNA and p-azido phenylalanyl-tRNA synthetase (Chin et al. 2002) specific for p-azido phenylalanine. The system contained p-azido phenylalanine (Bachem) in addition to all 20 natural amino acids. A mixture of reduced and oxidized glutathione was added supplementary for the synthesis of the single chain antibody fragment AntiEC5218 to maintain an oxidizing environment for the formation of disulfide bonds. Following protein synthesis the reactions were desalted two times against PBS using gel filtration spin columns to remove low molecular weight components as non-incorporated amino acids. Aliquots of desalted reactions with the p-azido phenylalanine containing proteins were incubated with fluorescent dye (DyLight650, Pierce, Thermo Scientific) accomplishing Staudinger ligation (Prescher et al. 2005) and desalted using NAP5 columns against PBS.

A detailed description of this orthogonal cell-free system and the whole procedure will be published shortly (manuscript in preparation). Further informations about the used systems are available at RiNA GmbH and NanoTemper GmbH.

### Protein Quantification

Protein concentrations were calculated based on a reaction containing radioactively labeled leucine ( $^{14}\text{C}$ -leucine) that was performed in parallel and subjected to hot TCA precipitation. A BSA standard was used in addition to control the calculated amount of protein via gel electrophoresis and coomassie staining.

### Assay conditions

All synthesis reactions had been gel filtrated against PBS. Calmodulin was kept constant at a concentration of 5 nM in both experiments, while Anti EC5218 was kept at a concentration of 25nM. The ligands,  $\text{Ca}^{2+}$ , M13-Citrine, M13 (mutant)-Citrine and Cyclophilin A were diluted in PBS buffer containing 0.025 % Tween-20 for the dose response experiments. The measurement of the interaction between Calmodulin and M13-Citrine was carried out in the presence of 10  $\mu\text{M}$   $\text{CaCl}_2$ .

### Instrumentation

The measurements were done on a NanoTemper Monolith NT.115 instrument.

For the interaction of Calmodulin with  $\text{Ca}^{2+}$ , the measurement was performed at 80 % LED and

40 % MST power. For the interactions between Calmodulin and the M13 peptides 20 % MST power were used and for that of AntiEC5218 and Cyclophilin 80 % MST power were used. The Laser-On time was always set to 30 sec, the Laser-Off time 5 sec.

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