FAQ: How do I determine the stoichiometry of my interaction?

What are the prerequisites?
Which experiments should be performed?
How do I derive the stoichiometry from those experiments?

Under certain conditions, MST can not only be used to determine the affinity of molecular interactions, but can also be utilized to obtain binding stoichiometries (the ratio of ligand to binding partner in the complex).

Prerequisites: The exact concentrations of fluorescent binding partner and ligand as well as the K_d of their interaction needs to be determined in order to design the stoichiometry experiment. As a rule of thumb, the concentration of your fluorescent molecule should be **at least 20-fold higher** than the K_d of the interaction (e.g. if the K_d is 100 nM, use at least 2 µM of fluorescent molecule).

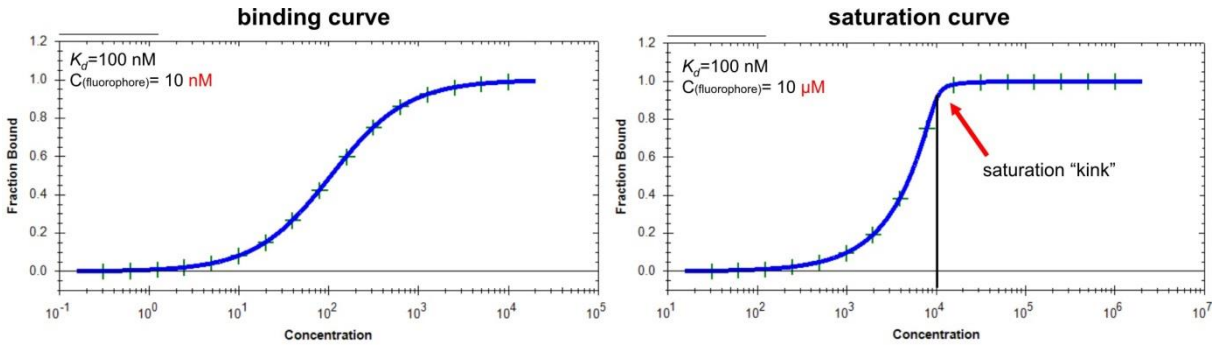

Figure 1: Simulations of a typical binding curve (fluorophore concentration < K_d) and a typical saturation curve (fluorophore concentration >> K_d) using the concentration finder tool implemented in the NT software. The saturation curve displays a characteristic "kink" (red arrow) at the ligand concentration at which the binding partner is saturated. In the shown example, saturation of 10 µM fluorescent molecule occurs with 10 µM ligand (black line), which corresponds to a 1:1 interaction. Please note that exact stoichiometry information cannot be directly extracted from the curves in this figure but require more detailed experiments and analysis as described below.

At fluorophore concentrations **below the K_d**, the titration experiment yields a sigmoid **binding curve (Figure 1, left).** In this case, the MST signal at each ligand concentration is determined by the ratio of bound to unbound ligand, and can thus be used to calculate the dissociation constant. However, a binding curve **cannot be used to determine the stoichiometry of the interaction**.

At fluorophore concentrations **above the K_d,** the titration experiment yields a **saturation curve (Figure 1, right).** Added ligand is completely bound to the fluorescent molecules until they are saturated. Once saturation is reached, a characteristic "kink" appears, and further addition of ligand does not affect the signal. **Saturation curves cannot be used to determine the K_d, but yield the stoichiometry of the interaction**, which is represented by the ratio of ligand concentration where the "kink" occurs and the concentration of fluorescent molecule.

Approach

The best practice for determining the stoichiometry of a molecular interaction using MST will be illustrated through the example of binding of biotin to streptavidin. The K_d of this interaction is extremely low (in the pico- to femto-molar range). Streptavidin is a stable tetramer, and each subunit is known to bind one biotin molecule.

1. **Determine concentration range for stoichiometry experiment (figure 2, left):**
   In an initial experiment, a **standard titration** using 25 nM fluorescently labeled streptavidin against biotin at concentrations ranging from 10 µM to 0.3 nM using a 1:1 dilution series was performed. Note that the streptavidin concentration is well above the K_d. This initial experiment already gives a first hint about the potential stoichiometry, as the saturation "kink" occurs around ligand concentrations of ~100 nM. However, since a 1:1 dilution series is used, **it is necessary to narrow down the ligand concentration range in order to obtain sufficient data points for a precise determination of the interactions' stoichiometry.**

2. **Stoichiometry experiment using a narrow concentration range (figure 2, right):**
   In a **second experiment**, the same streptavidin concentration is titrated against biotin concentrations in the range of the saturation point determined in the first experiment (in this case from 10-160 nM). **A 1:1 dilution series is no longer used in this case, and different ligand concentrations should be pipetted separately.** Ideally, there are less data points in the saturated part of the curve (5-6 points) and most data points in the non-saturated part (10-11 points). Note that the data in the example below are plotted on a logarithmic x-axis for the initial experiment (left), and on a linear x-axis for the second experiment (right).

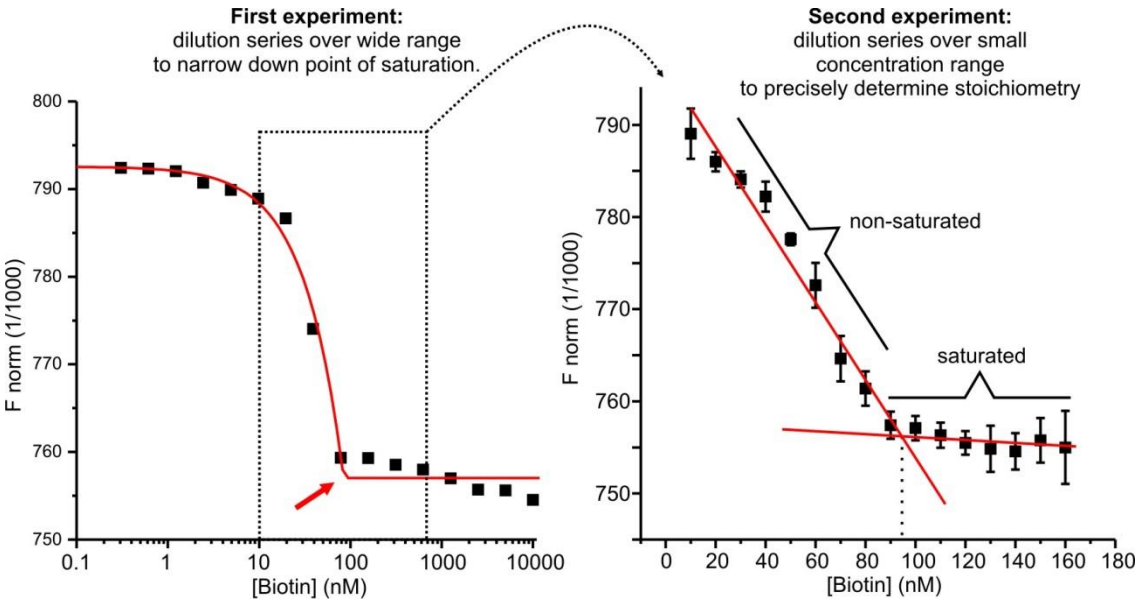

Figure 2: Determination of the binding stoichiometry of biotin and streptavidin. (Left) 25 nM labeled streptavidin were titrated against a wide range of biotin. The K_d fit yields a saturation curve (red line) with a "kink" at a biotin concentration of ~100 nM (red arrow). (Right) In a follow-up experiment, 25 nM labeled streptavidin were titrated with biotin in a narrow concentration range from 10-160 nM. Linear regression of the saturated and non-saturated parts of the data points reveals a saturation of streptavidin at 94.35 nM biotin, yielding a binding stoichiometry of ~ 4:1.

The stoichiometry of the interaction can now be analyzed. For this, the exact position of the saturation “kink” should be determined. It is recommended to plot the data on a linear x-axis to identify the linear sections of the saturated and non-saturated part of the curve. After linear regression of the respective data points, calculate the x-position of the section (corresponding to the ligand concentration at which saturation occurs) of the two linear regressions using:

$$x = (b_2-b_1)/(m_1-m_2)$$

where m is the slope, and b the y-intercept (according to the general equation y=mx+b)

For this particular example, the saturation concentration was determined to be 94.35 nM. Given a concentration of 25 nM streptavidin, this means that each streptavidin tetramer binds to 3.78 biotin molecules, which is in good agreement with the known 4:1 interaction.

Notes:

- Determination of interaction stoichiometry requires rather high affinities. For **low affinity interactions** with K_ds in the intermediate or high µM range, stoichiometry experiments are difficult since very high concentrations are required (e.g. for an interaction with a K_d of 10 µM, concentrations of ~100 µM of fluorescent molecule are required for reliable stoichiometry experiments).

- We recommend using the “concentration finder” tool which is implemented it the NT.Analysis and NT.Control software in order to determine suitable concentrations of fluorescent molecule and ligand.

- A stoichiometry < 1 can be caused by partially unfolded or inactive protein. In this case use a new protein batch or different buffer conditions. For buffer optimization please consult our Monolith Starting Guides.