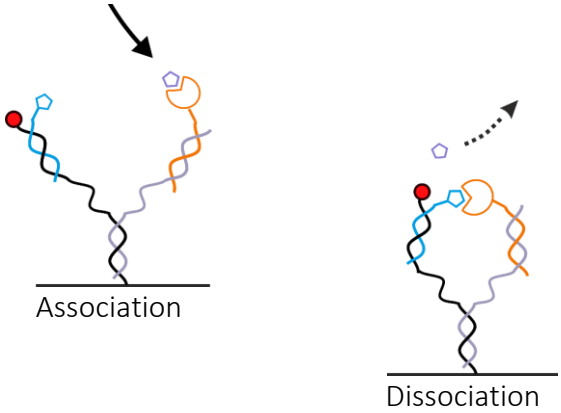
# DK-Kinase: Protein – Small Molecule ($k_{ON}$ | $k_{OFF}$ | $K_D$)

## Association and Dissociation of a Small Molecule

### Aims

- Familiarization with the software tools for performing switchSENSE® experiments
- Determination of the association and dissociation rates of a small molecule ($k_{on}$, $k_{off}$, $K_D$)




### Workflow

| | | |
|---|---|---|
| Design your experiment | **switch**BUILD | Step-by-step guidance through the workflow |
| Perform your experiment | **switch**CONTROL | Finetuning of the experiment (if needed) |
| Analyze your results | **switch**ANALYSIS | Simple drag´n´drop format for efficient data interpretation |

## Product description

**Product Code** DK-KS-1-B96

**Measurement Time** ~2 h 04 min

In this experiment, the binding of the small molecule Staurosporine to the Kinase PKA is analyzed. We start with immobilizing the Y-structure on the biosensor surface. Next, the association and dissociation of three consecutive concentrations of Stauroporine to PKA is observed.

**Before getting started, transfer one aliquot of each sample listed in Table 1 to the fridge to ensure slow thawing.**

TABLE 1 | Contents and storage information

| Material | | Cap | Amount | Storage | Comments |
|---|---|---|---|---|---|
| cNL-A96 / cNL-B96 (500 nM each) | | blue | 3 x 40 µL | -20°C | in PE40 |
| Y-structure-PKA (200 nM) | | green | 3 x 40 µL | -20°C | in TE40, 0.5 mM TCEP |
| Staurosporine | 5 nM | red | 3 x 500 µL | -20°C | in Running Buffer |
| | 10 nM | | | | |
| | 20 nM | | | | |

TABLE 2 | Additional material required

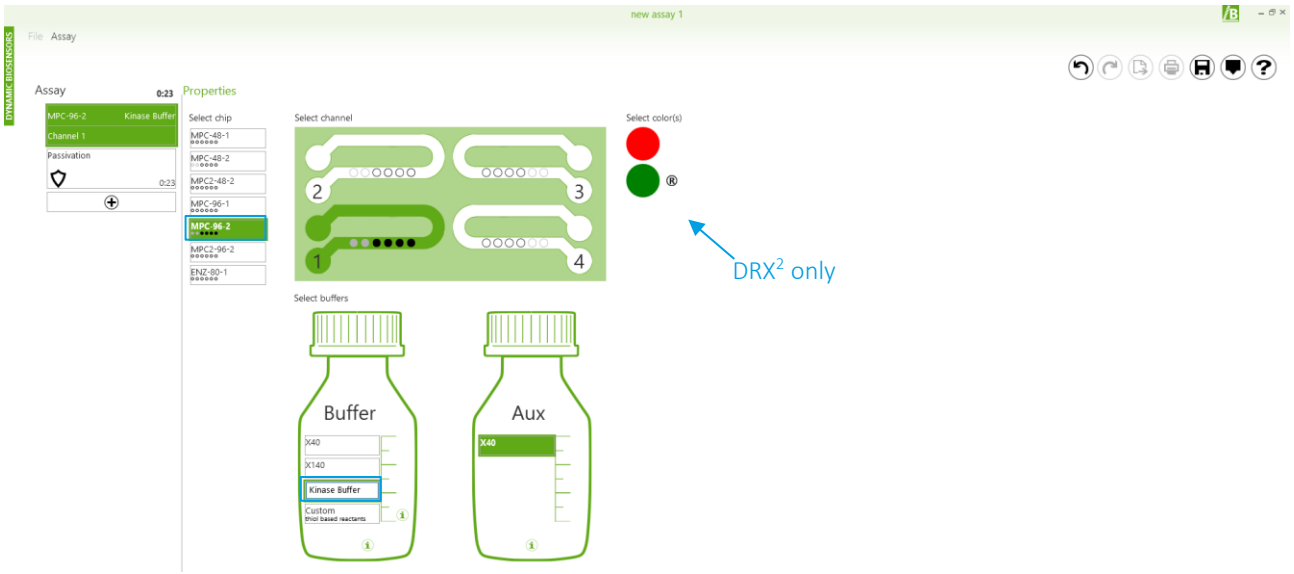
| Material | Cap | Amount | Storage | Comments |
|---|---|---|---|---|
| MPC-96-2 biochip (red amplitude > 400 kcps) | - | 1 | 2-8°C | use in DRX or DRX$^2$ |
| Kinase Buffer (1x):<br>10 mM TRIS-HCl, pH 7.4,<br>40 mM NaCl, 2 mM $MgCl_2$, 1 % DMSO, 50 µM EDTA,<br>50 µM EGTA, 0.05 % Tween 20, sterile filtered | transparent | 2 x 50 mL | 2-8°C | Running buffer |
| TE40 buffer (1x):<br>10 mM TRIS-HCl, pH 7.4, 40 mM NaCl, 50 µM EDTA,<br>50 µM EGTA, 0.05 % Tween 20, sterile filtered | transparent | 2 x 50 mL | 2-8°C | Aux buffer |
| DI Water | transparent | 1 x 12 mL | 2-8°C | |
| Passivation solution (10x) | yellow | 1 x 1 mL | 2-8°C | dilute in 9 ml DI water |
| 1x Regeneration solution | transparent | 1 x 12 mL | 2-8°C | |

Large glass vials (10 mL), small glass vials (1.5 mL), caps required. For *in vitro* use only.

## Planning the experiment

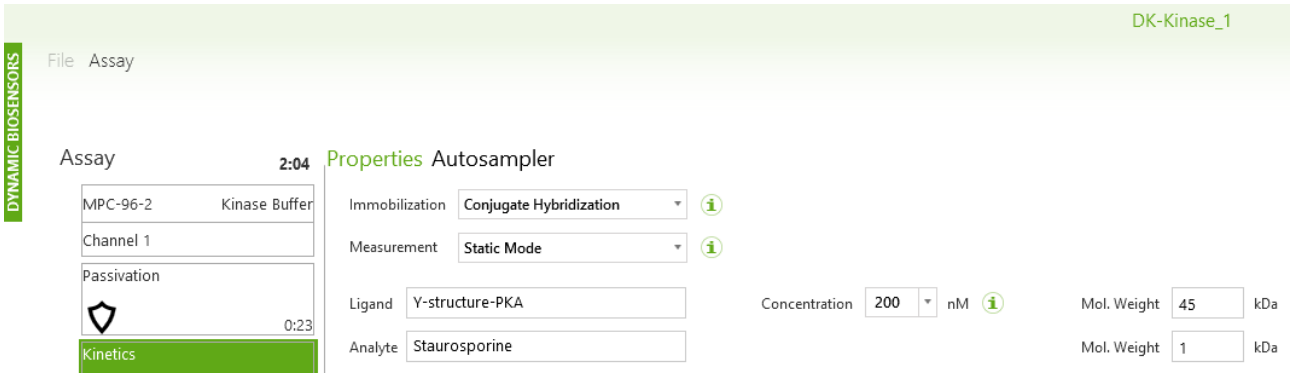
1. Start switchBUILD by clicking on the icon on the desktop and click on **+ New** to create a new assay. The interface is arranged in two different columns: *Assay* and *Properties*. The *Assay* column shows all elements of you assay. By default, it includes a panel to choose the basic settings of your assay and a *Passivation* element, which should be run before the measurement to prevent unspecific binding to the biosensor surface. Next to *Assay* the estimated runtime of your assay is shown. In the *Properties* column, the individual measurement parameters for each assay element can be set. The info button ⓘ always provides further information about the current topic.

2. Now, let's start with defining the general *Properties* of your assay by selecting the first panel of your assay. Choose the **flow channel** you want to run the experiment in by clicking on the channel – the selected channel is highlighted in green. Furthermore, the running buffer *Buffer* and auxiliary buffer *Aux* can be specified. For this demo select **"Custom"** as *Buffer*. You can enter your buffer composition if wanted. To specify the type of Biochip used, select "**MPC-96-2**" from the *Select chip* list, which indicates that it is a Multi Purpose Chip (MPC) with a functionalized DNA-length of 96 base pairs and two different DNA sequences. The two sequences are spatially separated with sequence A on electrodes 1 and 2, and sequence B on electrodes 3-6. You can use within one measurement sequence A as control and sequence B for the specific sample of interest.



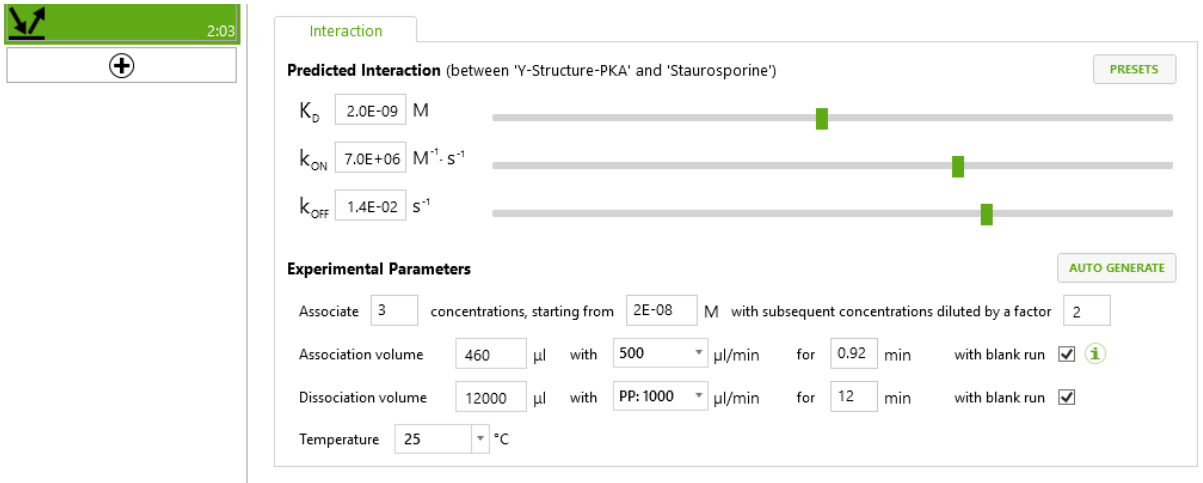

3. To add assay elements to the sequence, click ⊕ in the *Assay* column and choose the desired element. As the goal of this experiment is to determine binding kinetics, we need a **Kinetics** element.

4. In the next step, you should define the *Properties* of the Kinetics assay. In the upper section general information about your interaction experiment is requested. Select **Conjugate Hybridization** for *Immobilization*, which assumes that your ligand is conjugated to the complementary DNA. Change the *Measurement* type to **Static Mode**. In this mode, the DNA is kept in a defined upright position. Update the name of your immobilized *Ligand* to **Y-Structure-PKA**, since we are hybridizing the Protein Kinase A (PKA) conjugated to DNA oligos forming a Y-Structure, and select **200 nM** as concentration. The molecular weight of the kinase is around **40 kDa**. The name of your *Analyte* is **Staurosporine**; the small molecule has a molecular weight of **0.5 kDa**.



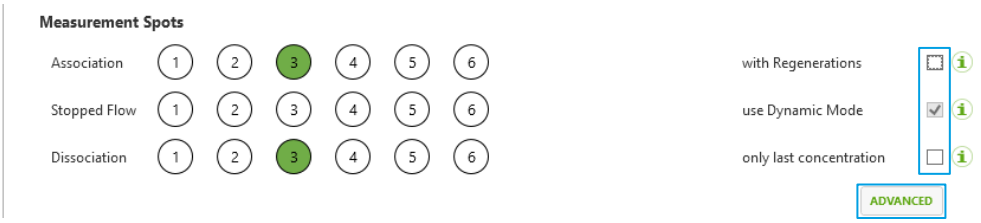

5. In the *Interaction* tab you are asked to give an estimation of the expected interaction parameters ($k_{on}$, $k_{off}$, $K_D$). You can either choose saved settings via PRESETS or enter custom values. Please enter the following parameters, either by moving the sliders or by typing the values in the corresponding fields: $K_D$ = **2.0E-9** M, $k_{on}$ = **7.0E+06** $M^{-1}s^{-1}$, $k_{off}$ = **1.4E-02** $s^{-1}$. When using the sliders, one of the three values will always be fixed, as each value is dependent on the other two: $K_D = \frac{k_{off}}{k_{on}}$. The values entered are used to predict the Fraction Bound [%] shown in the graph on the right, which is updated on the fly when you make changes in the next step:

6. As *Experimental Parameters* we keep **3** *concentrations* of Staurosporine, *starting from* the stock concentration of **2E-8 M**, which will be diluted by a *factor* of **2.** The resulting concentrations are displayed below the graph on the right. Additionally, add buffer control runs by activating the checkboxes next to **with blank run** for both Association and Dissociation. Next enter the *Association volume* of **460 µL**, with a pump rate of **500 µL/min**. The time is the result of volume divided by pump rate and can be used for setting the volume as well. The *Dissociation* should be run with **12000 µl** at a pump rate of **1000 µl/min** with the peristaltic pump (PP). If there is only a syringe pump (SP) available, you can use 2400 µL with a flowrate of 200 µL/min for dissociation.




7. In the third section, you are asked to select the measurement spots that will be used in your experiment. The selected spots are colored in green. For this demo, choose **one measurement spot** out of spots 3-6 and select it for both *Association* and *Dissociation*. **Deselect** all electrodes for the *Stopped Flow*.

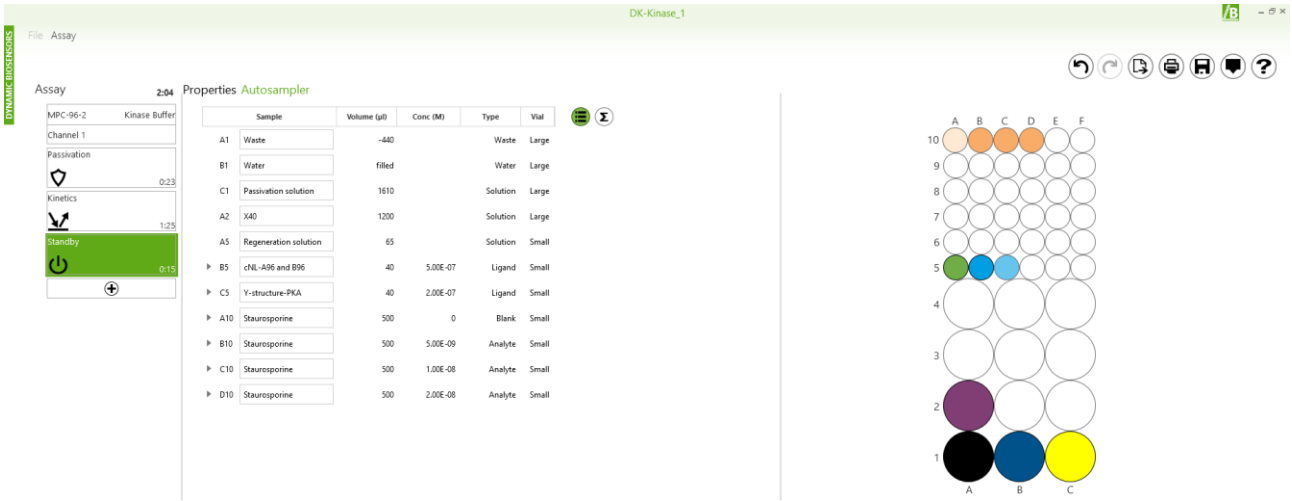
In addition, you can decide if the flow channel should be functionalized with fresh ligand after each concentration of analyte. This includes a regeneration, during which the biochip is washed with a proprietary regeneration solution (SOL-REG-12-1) to ensure a fully single-stranded state of all covalently immobilized DNAs. Subsequently, fresh conjugate DNA is re-hybridized. It is advisable to perform regenerations between concentrations if no complete dissociation of the analyte has taken place beforehand. Since Staurosporine is dissociating within 10 - 15 min, instead of regenerating we will fully dissociate each analyte concentration. To do so, please **uncheck** the two checkboxes *with Regenerations* and *only last concentration*. To improve the signal-to-noise ratio, you can increase the excitation power by clicking on ADVANCED and selecting the excitation power **2 LED** for *Association* and *Dissociation*.




8. At last, add a **Standby** assay element to your *Assay* sequence and choose **Reset surface** only. This step removes the ligand, functionalizes the chip with bare DNA, fills the measurement channel with air and thereby prepares the biochip for storage after your measurement.


9. Save your newly designed assay as a DBB file by clicking on and name the file "**DK-Kinase_1**".

10. You will be taken to the *Autosampler* tab, where you can see how the autosampler of the DRX should be loaded for the experiment. In the middle column you can find a list of all solutions and their concentrations needed for your measurements. When you click on an item in the solution list, its respective position in the autosampler display (right column) is indicated with a green circle.

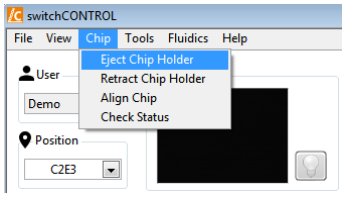



**Congratulations, you just designed your own switchSENSE® experiment!**

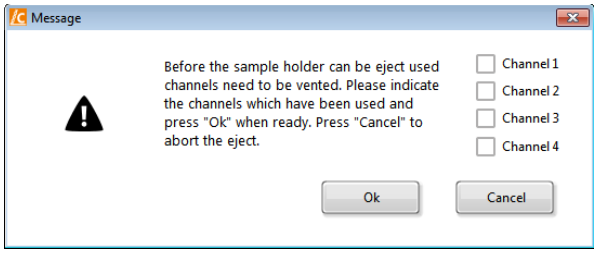
## Conducting the experiment

1. **Start switchCONTROL** by clicking on the icon on the desktop and enter **your name** when prompted (the measured data will be automatically saved in a folder with your name).

2. To insert a new chip, click on **Chip** → **Eject Chip Holder** in the upper left corner of the software window to eject the chip holder from the DRX.




3. The following message will pop up. Indicate which channels have been used on the inserted biochip by checking their respective checkboxes, and then click **Ok**.

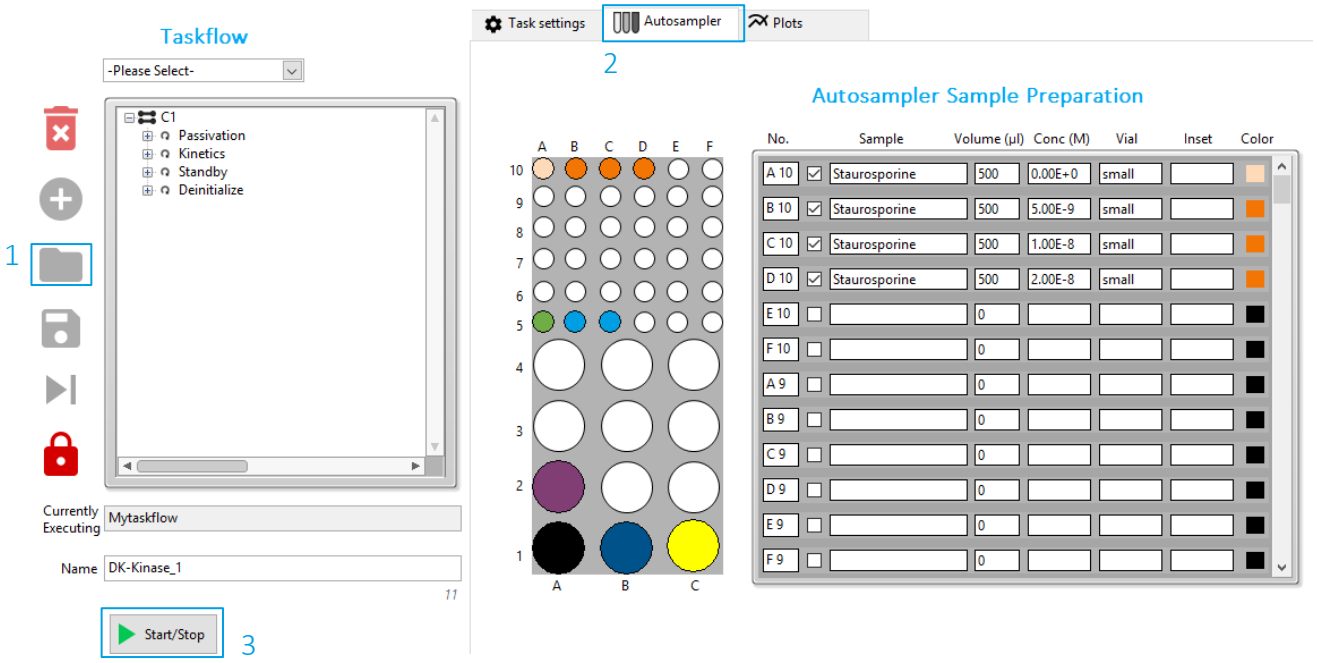



4. After venting, the chip holder will be ejected. Open the chip holder by pushing both white sliders on the right to the back. Use the vacuum pen to remove the chip currently in place and insert your **MPC-96-2** chip. Make sure to place it with the label facing upwards. Once the chip is in place, close the chip holder while pushing the white slider back.

5. Click on **Chip** → **Retract Chip Holder** to retract the new biochip into the DRX and follow the instructions of the **Retract assistant**. Enter the Chip-ID as shown on the packaging of your biochip. A message will pop up asking if the chip should be **aligned** – the alignment is necessary to precisely position the electrodes for measurement. An alignment should be performed every time a chip is placed into the DRX (except for a Cleaning chip which is not used for measurements). The next message will ask you if you wish to **prime** the microfluidics system with the running buffer – the priming should be done if this is your first measurement of the day or if your running buffer is different from that of the prior measurement. Make sure to connect the *Running Buffer* (Kinase Buffer) to the **Buffer** and the *Aux Buffer* (TE40 buffer) to the **AUX** reservoirs on the top left (exterior) of the instrument. Finally, you are asked if you want to check the status of the electrodes and regenerate the channel before starting the experiment. These steps are not necessary for the purposes of this demo and the default settings can be maintained. The alignment and priming will take a few minutes. You can follow the alignment by watching the small camera window on the top left of the software window, where you will see the focusing on the four alignment crosses.

6. While the alignment and priming are taking place, please prepare the samples as indicated in switchBUILD.

7. **Load your assay** *DK-Kinase_1* into switchCONTROL by clicking on at the left of the software window {1}. Next, click on the **Autosampler** tab {2} to view the sample positions listed within the script, and load the sample solutions into the autosampler accordingly. Close the autosampler door and start the measurement by clicking on Start/Stop {3}.



## Measurement Steps

If working with a DRX² DualColor instrument, choose to display the **red dye only** by clicking on the color block next to *Display* above the measurement plots Display .

### - Passivation and Status

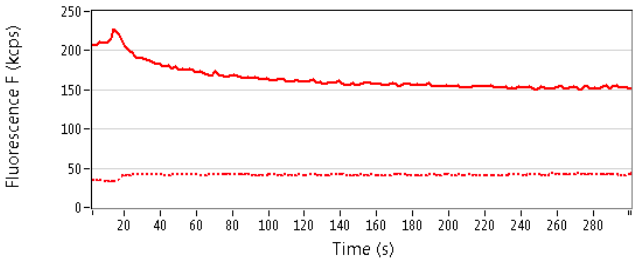
Before starting an experiment, the biosensor surface needs to be passivated to prevent unspecific surface binding, and thus enable maximal DNA-switchability. During the passivation step, the chip is incubated in passivation solution and the DNA levers are switched with a frequency of 0.2 kHz. Positive potential leads to a low fluorescence signal (as the fluorophore lies close to the surface), while negative potential leads to a high fluorescence signal (as the fluorophore is positioned away from the surface). The *passivation* step takes 10 minutes and should be run completely if the channel had not been used on the same day. If the channel has already been used, you can skip this passivation step after a few minutes by clicking on . To do so, the taskflow needs to be unlocked first by clicking on .

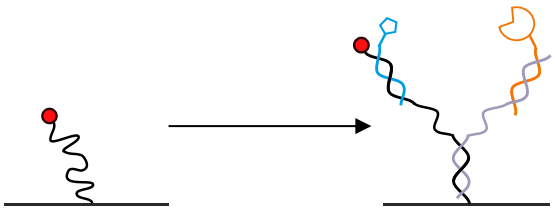
After the passivation the chip will be subjected to a *status* diagnosis: Each electrode is calibrated (The change of fluorescence intensity in response to the applied voltage is recorded and the inflection point determined. More positive voltages attract the negatively charged DNA and orient the DNA nanolevers closer to the quenching gold surface.) and the DR and Fluorescence values are recorded. Use this information in case electrodes need to be changed.

Please make sure to use an electrode, for which the fluorescence amplitude is above 400 kcps.

### - Functionalization

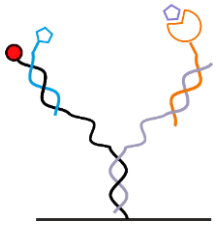
You will be able to follow the functionalization of the chip with the PKA-conjugated Y-structure in real-time in the **red channel**. Before the *functionalization*, the dsDNA is denatured by the injection of a *regeneration* solution (high pH), therefore the measurement starts with the switching of ssDNA. After a short while, a mix of the fresh oligos is injected into the flow channel and the Y-structure is formed. As dsDNA is more rigid than ssDNA, the fluorophore is pushed away further from the surface during switching, which normally leads to a fluorescence increase. In the case of the Y-structure, a competitor compound is hybridized next to the dye (blue), which efficiently quenches the fluorophore (fluorescence decreases). Since this effect is more pronounced than the fluorescence increase caused by elongation of dsDNA, the overall fluorescence decreases during hybridization. The functionalization takes about 15 minutes for completion.



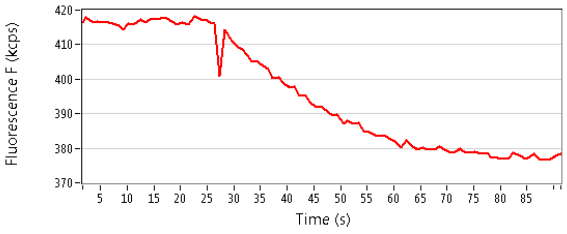
### - Association and Dissociation of Staurosporine

The binding of Staurosporine to PKA is measured in Static Mode, which implies that the DNA nanolevers are not actuated but kept in an upright position. Since without switching of the DNA nanolevers no Dynamic Response can be determined, the plot for Dynamic Response is only a flat line. For the same reason, only the Fluorescence in the top position is plotted below.

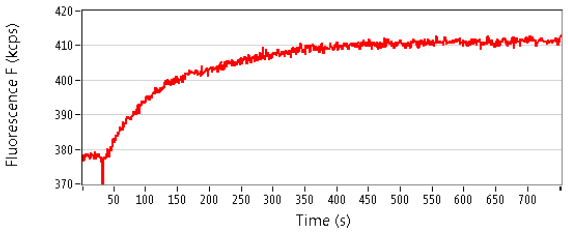


For 30 secs, the fluorescence intensity of the nanolevers functionalized with PKA will be measured. After that, the Staurosporine solution is injected. Now you can follow the binding of the small molecule to PKA in real-time: The quenching effect of the immobilized compound (blue) is strongest, if it is not bound to the kinase (orange). That state is induced by the analyte, here Stauroporine (grey), when it associates to the kinase. Thus, a decrease in fluorescence can be observed until equilibrium is reached. After each concentration, running buffer is injected into the microfluidic channel. You can follow the dissociation of the small molecule in real-time by an increase in fluorescence. Since Staurosporine dissociates almost completely within a few minutes, no fresh conjugate is immobilized between the different concentrations of Staurosporine.

Association



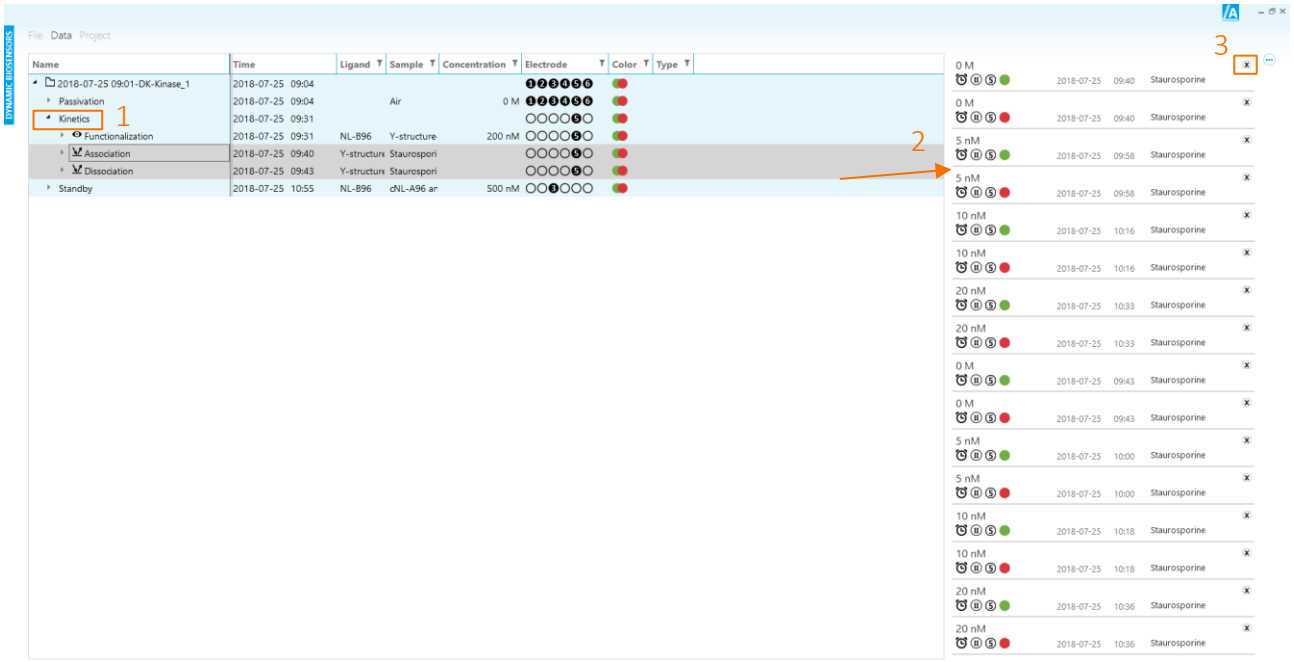

Dissociation



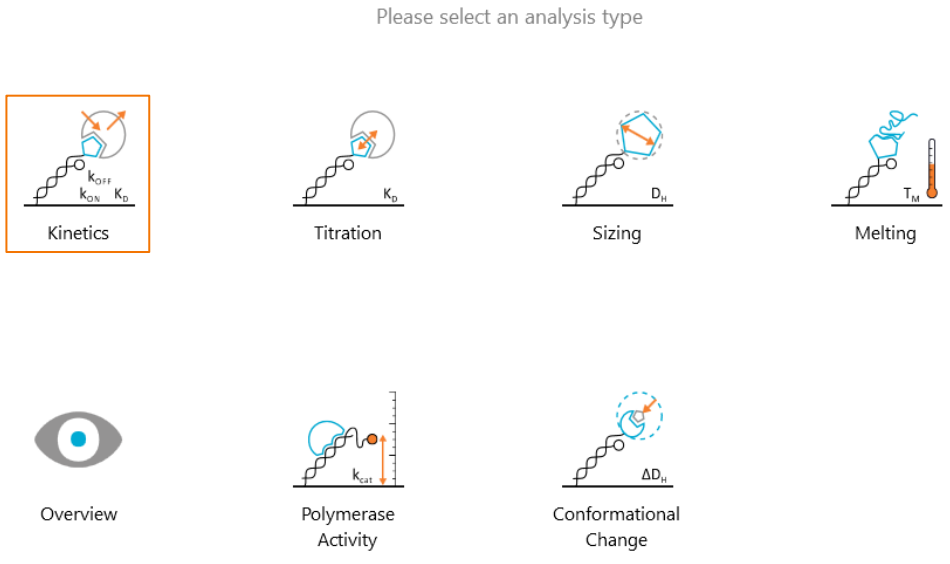
## Data analysis with the switchANALYSIS software

1. Start switchANALYSIS from the icon on the desktop and click on New to create a new assay. Now you can load your data by clicking on **IMPORT EXPERIMENT FILE** in the lower left corner and selecting your zipped measurement data, which is saved in > folder with your username > subfolder with the date.

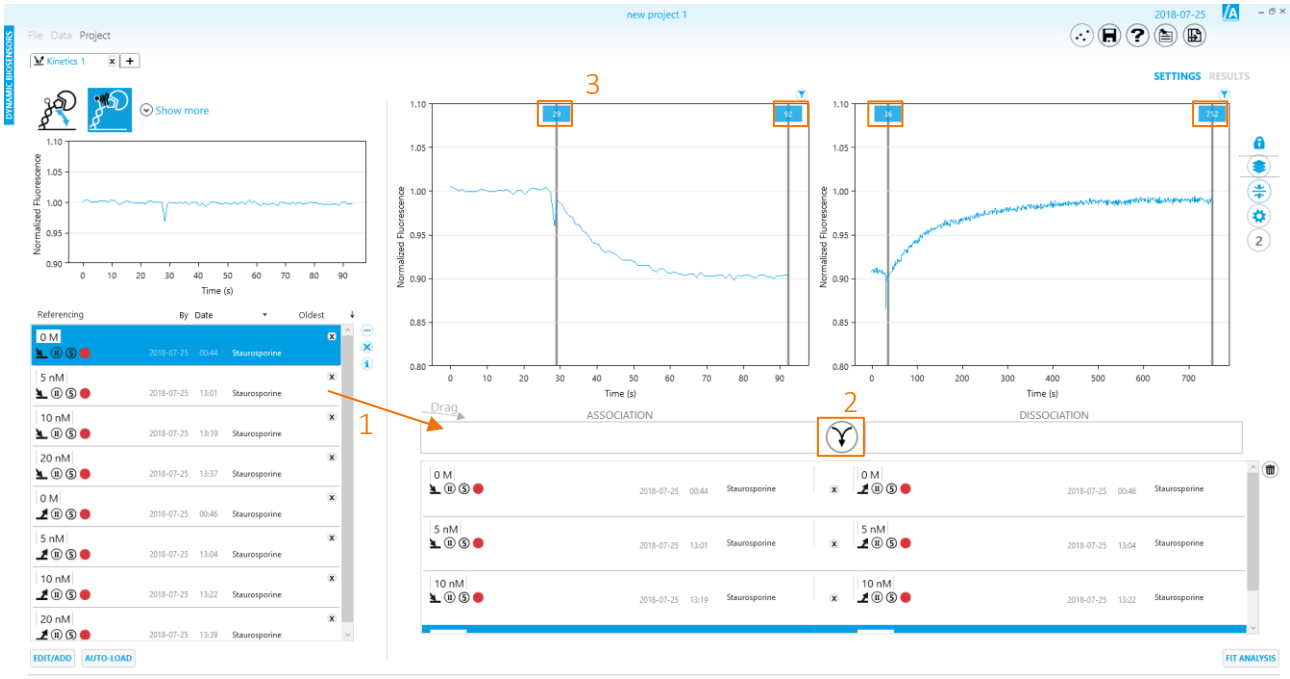
2. You will find the assay structure of your switchBUILD script (Passivation – Kinetics – Standby) when expanding the measurement file (indicated by the start time of your measurement) by **clicking on the triangles**. Further expansion of the *Kinetics* dataset {1} reveals data for the Functionalization and the Association and Dissociation of Staurosporine. Drag-and-drop the folders **Association** and **Dissociation** into the right column to select the data {2}. Both the red and green channel recorded data during the measurements, however, we did not use any green-labeled DNA, therefore **delete all green** marked data with {3}. You can also mark all green measurements in the right column by pressing *Crtl* on the keyboard while selecting and delete them simultaneously with *Del*.




3. Click on **CREATE NEW ANALYSIS** in the lower right corner and select the **Kinetics** analysis type to analyze the kinetic rates.



4. On the SETTINGS page you will see the data you just imported. Association and dissociation should be paired automatically in the main column, or you can drag-and-drop them from the left column {1} into the respective box and add them with the middle button {2}. The fitting range is preselected automatically as well and indicated by the sliders. Select each measurement one by one in order to verify that the fitting borders are set accurately. They can be adjusted by entering a number after double-clicking on the value or by moving the sliders {3}. Proceed by clicking on **FIT ANALYSIS** in the lower right corner.




5. You will see a mono-exponential fitting curve for the association and dissociation data. To incorporate all data except the buffer control, the global mono-exponential fitting model is selected in the left column. One value for $k_{on}$, $k_{off}$ and $K_D$ is calculated from all curves.

6. Finally, you can save your analysis by clicking on .

**Congratulations, you are a switchSENSE® expert user now!**

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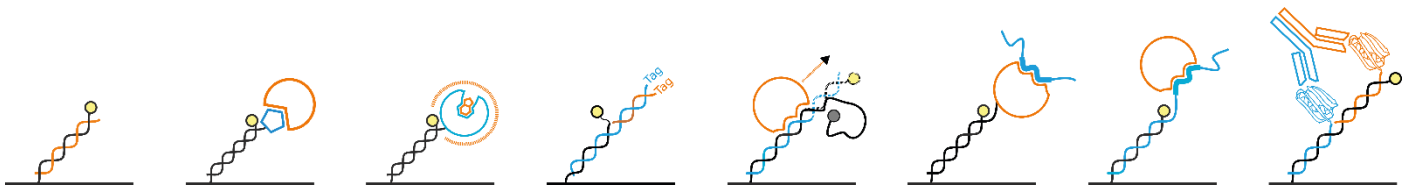

**switch**SENSE® is a proprietary measurement technology by Dynamic Biosensors GmbH.
Instruments and biochips are engineered and manufactured in Germany.