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Application Note

Repeatability of binding kinetics measurements and regeneration performance of a switchSENSE® sensor

Keywords: Repeatability I reproducibility I Fluorescence Proximity Sensing (FPS) I kinetics I affinity I regeneration I small molecule

The repeatability of binding kinetics measurements and the regeneration performance of a switchSENSE Multi-Purpose Chip (MPC) are presented in this Application Note. Association and dissociation kinetics of a small molecule analyte (desthiobiotin) and a protein ligand (streptavidin) were measured with a DRX instrument. 40 subsequent runs including association and dissociation phases and surface regeneration steps were performed on one measurement spot, i.e., the same microelectrode. The evaluated association and dissociation rate constants k_{ON} and k_{OFF} were found to be highly reproducible with a coefficient of variation of less than 3 %.

Methods and Results

A standard switchSENSE Multi-Purpose Chip (Dynamic Biosensors, MPC-48-2-Y1-P) with 48 bp DNA nanolevers was used for the experiments. Measurements were performed with a single-color (yellow) DRX instrument.

To evaluate the repeatability[1] of binding kinetics measurements, 40 measurement cycles were subsequently performed on one and the same detection spot. Each cycle consists of three steps which are schematically shown in Figure 1:

Step 1 – Functionalization: Single stranded DNA nanolevers on the Multi-Purpose Chip were hybridized for 10 minutes with a DNA-streptavidin conjugate. Ready-to-use conjugate is available from Dynamic Biosensors (CK-SA-1-B48); alternatively, streptavidin (e.g. Thermo Fisher Scientific, 21122) can be covalently coupled to complementary DNA using an amine coupling kit (Dynamic Biosensors, CK-NH2-1-B48). The concentration of the DNA-streptavidin conjugate used for the functionalization step was 250 nM; lower (higher) concentrations could be used as well, which would result in longer (shorter) hybridization times. Hybridization kinetics were measured in TRM (0.2 kHz).

[1] The term “repeatability” refers to the agreement of measurements that are performed by the same scientist using the same set of samples (molecules, chips) and the same instrument in a short period of time. By contrast, “reproducibility” refers to the agreement of measurement results obtained by different scientists at different times and locations, using different machines of a same type of instruments, and using different production batches of the same type of samples and chips.

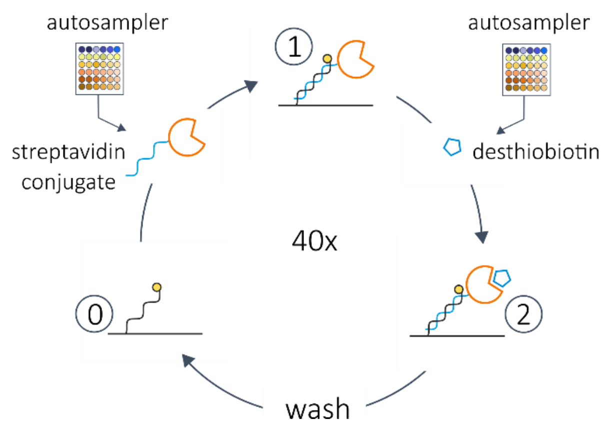

Figure 1 | Measurement cycle. Sensor functionalization (0-1): First, single-stranded DNAs bound to the measurement electrode are functionalized with streptavidin by hybridizing complementary DNA-streptavidin conjugates to the surface. **Binding kinetics measurement (1-2):** Second, the association and dissociation kinetics of solute desthiobiotin to immobilized streptavidin are monitored in real-time using the Fluorescence Proximity Sensing mode. **Washing (2-0):** Third, the DNA-streptavidin conjugate is removed from the surface by denaturing the DNA nanolevers at basic pH. The sensor is now ready for the next measurement cycle. Overall, 40 cycles (runs) were carried out on one measurement spot.

Step 2 – Binding Kinetics: Association and dissociation of desthiobiotin (Sigma Aldrich, D1411) to and from immobilized streptavidin was monitored in real-time using the FPS (Fluorescence Proximity Sensing) mode. During the association phase 10 nM desthiobiotin dissolved in PBS buffer 'P40' (pH 7.4, Dynamic Biosensors, BU-P-40-20) was flown at a rate of 100 µl/min across the sensor surface for 5 minutes. Dissociation was measured for 10 minutes in P40 running buffer at a flow rate of 100 µl/min.

Step 3 – Washing: To remove used DNA-streptavidin conjugate from the sensor the surface was exposed to regeneration solution (Dynamic Biosensors, SOL-REG-12-1) for a few seconds. The high-pH regeneration solution denatures the DNA nanolevers by disrupting hydrogen bonds between the base pairs. The streptavidin conjugate is washed away while the covalently attached single stranded part of the nanolever remains on the surface and can be reused for a new functionalization (hybridization) step.

Real-time association and dissociation data were analyzed with the kinetics module of the switchANALYSIS software (v1.4, Dynamic Biosensors).

Results

At first we evaluate the **regeneration performance of a single measurement spot**. The stability of the fluorescence signal over the course of 40 measurement cycles is presented in Figure 2, which shows the fluorescence switching amplitude after sensor functionalization with DNA-streptavidin conjugate (cf. state 1 in Figure 1). The average decrease in fluorescence intensity is ca. -1 % per measurement cycle, however, a slightly higher decrease can be observed during the first few cycles. This small signal change results from a minor loss of DNA nanolevers from the surface during the regeneration step as well as gradual photo- and chemical-bleaching of the dye.

Overall, the signal is well-behaved and the electrode would allow for many more than the 40 performed measurements. As will become apparent below, the absolute intensity of the fluorescence signal is not crucially important, much rather, the quality of a binding kinetics measurement is determined by a good signal-to-noise ratio. In fact, the kinetic measurements reported below were performed using the auto-LED setting, which means that the illumination intensity is automatically adjusted before each measurement to compensate for changes in the

absolute fluorescence intensity from one regeneration cycle to the next. As a consequence, the decrease in fluorescence from run to run as

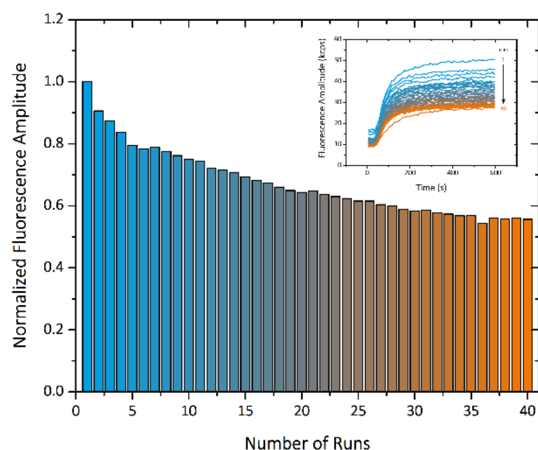

Figure 2 | Regeneration Stability. At the beginning of each measurement cycle the chip was functionalized with a fresh DNA-streptavidin conjugate. The fluorescence amplitude after the formation of the DNA-streptavidin nanolever is shown in the main graph. The inset shows raw data of real-time hybridization curves for 40 runs. The switching fluorescence amplitude increases when DNA-streptavidin conjugate hybridizes to the surface attached nanolever.

depicted in Figure 2 has no influence.

The **repeatability of a small molecule–protein interaction kinetics analysis** was investigated by measuring a total number of 40 association and dissociation curves, which are plotted in Figure 3 A&B. The fluorescence signal increases by 45 % upon binding of desthiobiotin (c = 10 nM) to immobilized streptavidin. This reversible fluorescence change results from the displacement of the fluorophore from the proximity of the biotin binding pocket, where the dye is significantly quenched.

In buffer flow (dissociation phase), the signal decreases by the same amplitude again, which indicates the complete dissociation of desthiobiotin from immobilized streptavidin. A similar behavior has also been observed for biotin, albeit at a much slower dissociation rate, which is why desthiobiotin has been used here for convenience.

The repeatability of the 40 measurements is very good, as can be seen from the excellent overlap of the curves. To quantify the interaction all association and dissociation curves were fitted with single exponential functions (Eq. 1).

$$F(t) = a \cdot \left(1 - exp\{-k_{on}^{obs} \cdot t\}\right) \quad (1a)$$
$$F(t) = a \cdot exp\{-k_{off} \cdot t\} \quad (1b)$$

The fit analysis yields the observable association rates k_{on}^{obs} and the dissociation rates k_{off} for each run.

The individual rate constants of the 40 runs are plotted in Figure 3 C&D and the average values as well as the standard deviation are summarized in Table 1. The coefficients of variation (COV = std. deviation/mean) for both rate constants are smaller than 3 %.

Table 1 | Statistical analysis of measured association and dissociation rate constants for the desthiobiotin-streptavidin interaction; n = 40, [desthiobiotin] = 10 nM.

	k_{on}^{obs} (10^{-2} s^{-1})	k_{off} (10^{-2} s^{-1})
Mean value	3.53	1.14
Standard deviation	0.075	0.032
Coefficient of variation	**2.1 %**	**2.8 %**

From the measured kinetic rates k_{on}^{obs} and k_{off} we can calculate the association rate constant (k_{on}) from $k_{on}^{obs} = c \cdot k_{on} + k_{off}$ and the dissociation constant $K_D = k_{off}/k_{on}$.

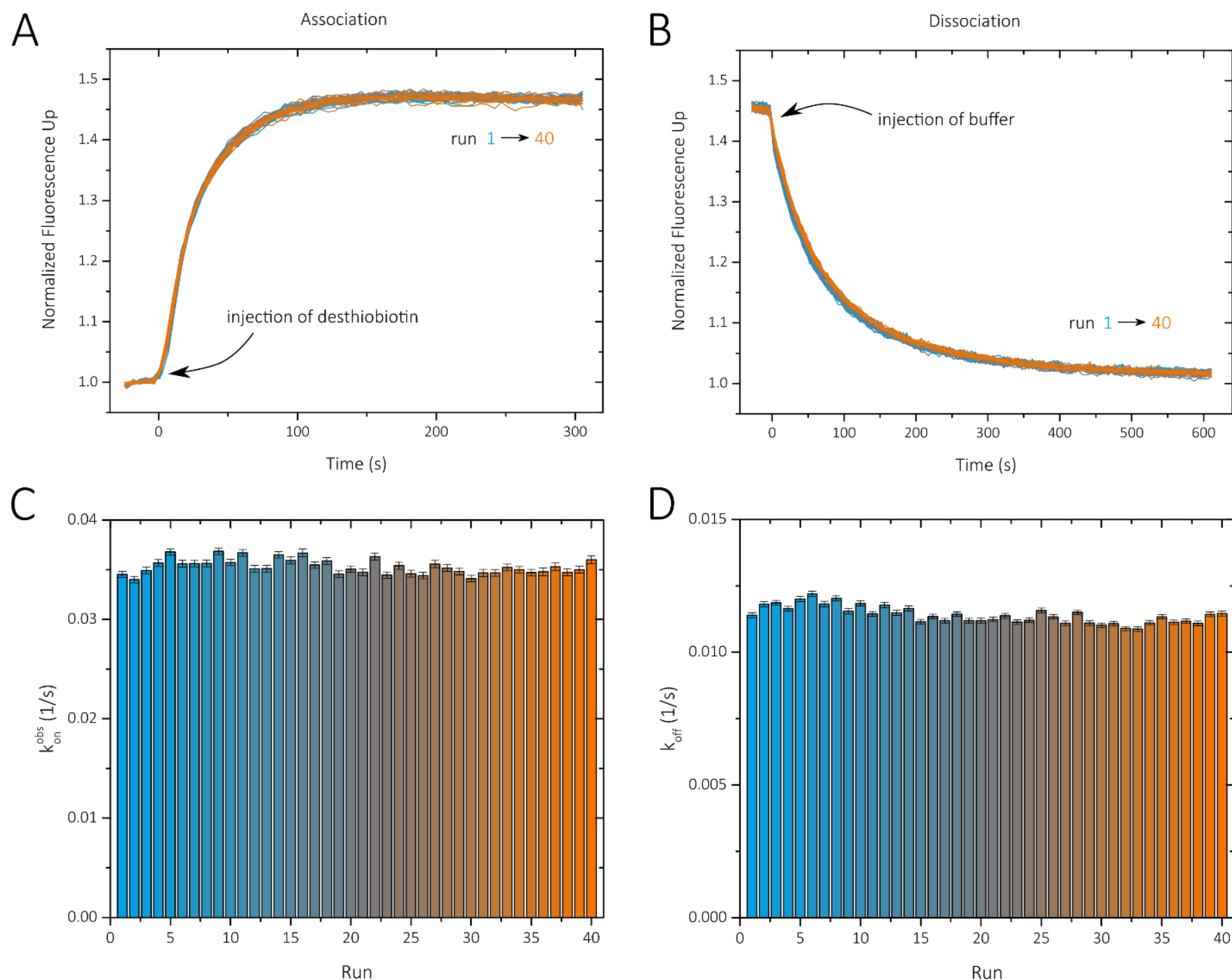

Figure 3 | Association and dissociation kinetics measurements for desthiobiotin binding to streptavidin immobilized on a switchSENSE multi-purpose chip. (A) 40 association measurements of 10 nM desthiobiotin binding to streptavidin. (B) Overlay of 40 dissociation measurements in buffer flow. Data in A and B are normalized to the fluorescence values measured before desthiobiotin injections. (C) Observable association rate k_{on}^{obs} and (D) dissociation rate k_{off} for each individual run. Rate constants were analyzed by least-squares fits of individual association-/dissociation-curves with single-exponential functions, Eq. 1 (switchANALYSIS v1.4).

Hence, we obtain for the desthiobiotin streptavidin interaction:

$$k_{on} \quad = \quad 2.39 \pm 0.02 \times 10^{6}\, M^{-1} s^{-1}$$
$$k_{off} \quad = \quad 1.14 \pm 0.03 \times 10^{-2}\, s^{-1}$$
$$K_D \quad = \quad 4.77 \pm 0.04\, nM$$

Conclusions

The analysis of 40 real-time binding measurements performed on the same detection spot shows that kinetics could be measured with excellent repeatability:

- The coefficient of variation for the determined on- and off-rates is less than 3 %.
- No systematic drift of the rate constants was observed over 40 repeated measurements.
- The binding kinetics data were of high quality, i.e., no indications for unspecific binding, mass-transport limitations, or rebinding were observed and all kinetics

followed simple single-exponential curve shapes.

- The average decrease in the absolute fluorescence intensity over 40 regeneration cycles was 1.1 % per cycle, however, this did not affect the analysis of the binding rates and was compensated by the auto excitation power setting.
- Even after 40 regeneration cycles, the measurement spot was fully functional and could have been used for more experiments.

The presented experiments demonstrate the robustness of the switchSENSE technology: a single detection spot was regenerated 40 times and the kinetic rate constants of a small molecule–protein interaction with a nanomolar K_D (desthiobiotin – streptavidin) could be analyzed with excellent repeatability.

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