Investigation of binding properties of high affinity monoclonal antibodies

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Abstract

The ConSense™ Analyzer has been used to study equilibrium dissociation constants in the nanomolar range. A pair of monoclonal antibodies was chosen as a test system in a homogenous and easy-to-use assay format. The data were analyzed with fluIT´s new accurate stochastic fluorescence spectroscopy (ASFS).

Materials and method

The tracer, Alexa532-labeled goat anti-mouse IgG (Invitrogen), was diluted with buffer to reach a final concentration of 0,5 nM and was reacted with mouse anti-human IgG (Invitrogen) at nine different concentrations ranging from 0.02 to 5 nM. The reaction buffer contained HEPES, EDTA, NaCl and SDS.

Sample preparation

Stock solutions of antibodies were diluted with reaction buffer in a final volume of 250 µl to reach the final concentrations, mixed and allowed to react to equilibrium at room temperature. 8µl of each mixture were transferred into ConSense Type A microchips. Each measurement was done in three replicates.

Instrumentation

Measurements were conducted with the confocal ConSense Analyzer using the 532nm single mode, single frequency laser for excitation. The sample were presented in the 14-channel standard detection chips (ConSense Type A). Parameter settings:

- sample volume: 8µl
- read time: 1 minute
- excitation power: 190 µW

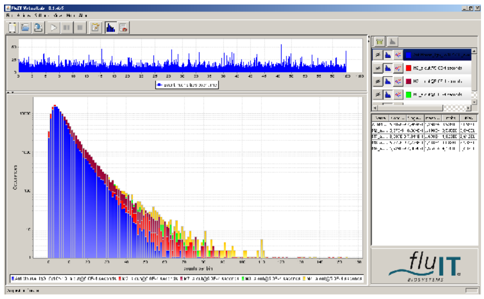

Fig. 1: Virtual lab screenshot for selected reads at different IgG concentrations

Results

The photon count distributions displayed in Fig. 1 clearly show a clear shift towards higher intensities, indicating the presence of molecules with higher brightness.

The blue data set represents the solution containing only the tracer, the upper line shows the peaks that are registered when fluorescent molecules pass through the observation volume of the ConSense Analyzer during a read of one minute.

An increase in molecular brightness occurs upon binding of mouse IgG to the tracer anti-mouse IgG. The tracer alone shows a maximum brightness of 24 kcps, whereas the complex [goat anti mouse IgG-mouse IgG] exerts a maximum brightness of 31 kcps.

This can be attributed to the fact that the complex is bigger and thus diffuses slower in the solution. In the focus of the laser beam these molecules will be more efficiently excited and thus yield a higher fluorescence signal making them nicely distinguishable from unbound tracer.

Both effects, change in diffusion time and brightness can be illustrated by plotting the maximum molecular brightness against the bin time (Fig. 2).

The turning point of the curves is a measure for molecule size and the molecule´s diffusion time. As can be seen from Fig.2, a certain shift can be observed in this titration series, but the curves are insufficiently separated to obtain quantitative results based on this parameter alone.

Further analysis reveals that a third, even brighter component (39,8 kcps) is present in a concentration range showing an excess of goat anti-mouse IgG. Fig. 3 shows the results of a three component fit. The nearly doubled brightness of this component and its higher diffusion time suggests that this component is a complex of two anti-mouse IgGs binding to one mouse IgG. At higher mouse IgG levels this component is replaced by the 1:1 complex, reflecting the stochiometry of both components in the solution.

	brightness (Kcps)	T_{Diff} (ms)
Anti-mouse IgG	23,84 ± 1,3	4,01± 0,8
[anti-mouse IgG -mouse IgG]	30,78 ± 1,0	7,04 ± 0,8
[2x anti-mouse IgG -mouse IgG]	39,81 ± 5,9	8,27 ± 2,4

Conclusion

Using the ConSense Analyzer the new ASFS is able to determine diffusion parameters, concentrations and binding constants directly from the measurement of molecular brightness without any assumptions on the laser profile and photo-physical properties of the molecules.

In contrast to other technologies based on fluorescence fluctuation (e.g. FCS) the ASFS technology is able to detect and quantify reactions of two reaction partners with equal size in solution making use of the additional information obtained by the simultaneous acquisition of brightness and diffusive measurement parameters.

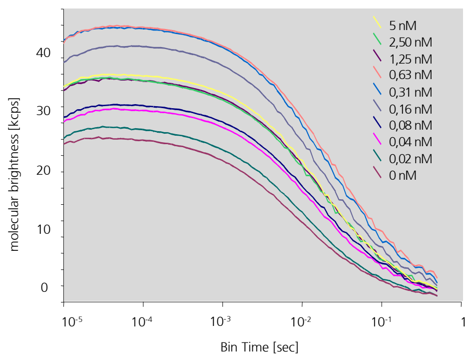

Fig. 2: Molecular brightness changes in the titration series displayed as a function of bin time. The legend shows the concentration of mouse IgG.

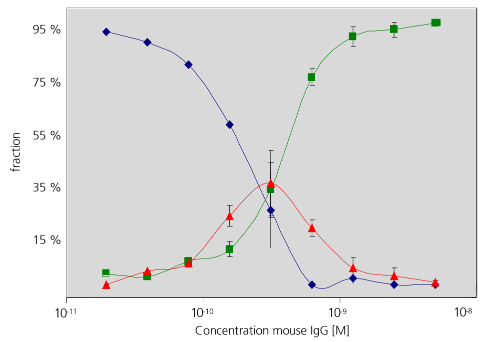

Fig. 3: The fractions of different molecular species as a function of mouse IgG, concentration (unbound goat antimouse IgG in blue; complex of two anti-mouse IgG and one mouse IgG in red, complex of one anti-mouse IgG and one mouse IgG in green, standard deviations of three replicates