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Precise quantification of transcription factors in a surface-based single-molecule assay

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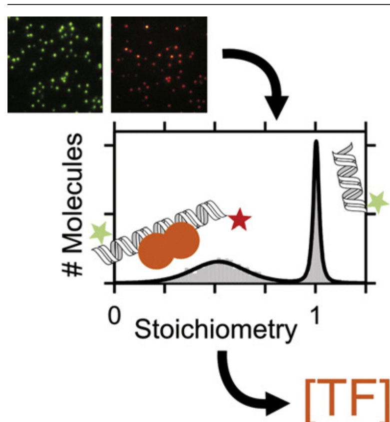
HIGHLIGHTS

- Single-molecule based assay for quantitative detection of transcription factors
- Improved quantitative modeling of data from single-molecule experiments
- Immobilized assay format reaches higher sensitivity than solution based approach.

GRAPHICAL ABSTRACT



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ABSTRACT

Biosensors have recognized a rapid development the last years in both industry and science. Recently, a single-molecule assay based on alternating laser excitation has been established for the quantitative detection of transcription factors. These proteins specifically recognize and bind DNA and play an important role in controlling gene expression. We implemented this assay format on a total internal reflection fluorescence microscope to detect transcription factors with immobilized single-molecule DNA biosensors. We quantify transcription factors via colocalization of the two halves of their binding site with immobilized single molecules of a two-color DNA biosensor. We could detect a model transcription factor, the bacterial lactose repressor, at different concentrations down to 150 pM. We found that robust modeling of stoichiometry derived TIRF data is achieved with Student's t-distributions and nonlinear least-squares estimation with weights equal to the inverse of the expected number of bin entries. This significantly improved transcription factor concentration estimates with respect to distribution modeling with Gaussians without adding notable computational effort. The proposed model may enhance the precision of other single-molecule assays quantifying molecular distributions. Our measurements reliably confirm that the immobilized biosensor format is more sensitive than a previously published solution based approach.

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1. Introduction

Biosensors have recognized a rapid development the last years in both industry and science. Although in the past decade the focus was

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on automation, in recent years it has shifted to miniaturization trying to integrate the detection of biomaterials with nanostructures [1]. An important parameter of miniaturization is whether the biosensor is amenable to surface-based immobilization. The new generation of biosensors must not only provide a qualitative answer on the presence of the analyte in question, but must also be capable of quantitative determination. Here, we set out to use immobilized DNA biosensors to quantify transcription factors (TFs), proteins that specifically recognize and bind DNA. TFs are pivotal molecules that control gene expression and their dysregulated profile is involved in numerous cancer types and diseases [2–4]. For this reason, their rapid and ultrasensitive detection is of essence in molecular diagnostics [5]. Long-established biochemical methods such as DNAse footprinting, electrophoretic mobility shift assays (EMSAs) and western blotting provide valuable information on characterizing TFs but are time-consuming, require large amounts of sample, and are at best semi-quantitative [6]. Enzyme-linked immunosorbent assays (ELISAs) and recent techniques such as the proximity-based ligation assay offer sensitive detection [6,7]. Their need for signal amplification renders them ill-suited for fast diagnosis, although their immobilization scheme offers a notable advantage for multiplexing and high-throughput capabilities.

Recently, TF detection with a single-molecule DNA biosensor was reported based on TF-induced coincidence of fluorescently labeled DNAs [8]. TF sensing of catabolite activator protein (CAP) and lac repressor (lacR; aka lacI), both involved in the regulation of bacterial lactose metabolism [9,10], was quantitatively investigated in solution using alternating-laser excitation (ALEX) and the robustness of the assay in cell extracts was demonstrated. Additionally, it was shown that in principle this assay also works with immobilized biosensors on surfaces. However, in order for the single-molecule DNA biosensor to be fully utilized in the immobilized scheme a thorough quantification and optimization is needed. Here, we continue the work published by Lymperopoulos et al. by precisely quantifying the fraction of lacR bound DNA using immobilized biosensors. We present an improved model for accurately describing the molecular distributions in stoichiometry-based single molecule experiments and adapted the established theoretical description of the biosensor response to surface-based measurements.

2. Results and discussion

2.1. TF sensing with DNA biosensors

The biosensors were constructed as described in ref. [8]. In brief, the biosensor comprises the TF–DNA target site split into two parts. These DNA fragments, termed half-sites (H), have short, complementary single stranded (ss) overhangs. In our assay, one half-site ($H1^G$) is labeled with a green fluorophore and biotinylated for single-molecule immobilization via biotin–neutravidin on a BSA passivated surface (see Materials and methods). The other half-site ($H2^R$) is labeled with a spectrally distinct red fluorophore and supplied in solution along with the TF (Fig. 1a). The tendency of the DNA fragments to associate is designed to be low such that in the absence of TF the fraction of annealed DNA half-sites is insignificant (Fig. 1c, bottom, Fig. 2a, bottom and Supporting Information Fig. S2a). We investigate lacR with a biosensor that has only 6 bases ss overhang and use low nanomolar concentrations of half-site $H2^R$ to satisfy this requirement. When the protein is present in the reaction mixture, it will selectively bind to the assembled binding site and drive the association of $H1^G$ and $H2^R$ (see Fig. 1b and c, top). We confirmed that lacR does not bind to one half-site alone with ensemble EMSAs (see Supporting Information Fig. S3). Protein-dependent colocalization of $H1^G$ and $H2^R$ is detected using ALEX on a total internal reflection fluorescence (TIRF) microscope. The fluorescently labeled half-sites of the single-molecule biosensor are sampled by alternating excitation with green and red laser lights. For each molecule in the green detection channel the average apparent Förster resonance energy transfer (FRET) efficiency (E^*) and stoichiometry (S) are calculated excluding unspecifically adsorbed $H2^R$ from the analysis (see Materials and methods). The assay readout does not require a change in FRET efficiency for TF sensing, but FRET may be used as an additional parameter for sensor encoding. Therefore, the S-based detection scheme allows a free choice of dye positions along the DNA, here at the respective ends of the half-sites to avoid perturbation of TF–DNA binding [8]. The interfluorophore distance is 40 base pairs (~13.6 nm) for the assembled half-sites of the lacR biosensor, corresponding to more than twice the Förster radius $R_0 = 6.2Å$ for the Cy3B–Atto647N dye pair, so FRET can be neglected in our experiments [11]. With $E^* = 0$ in the TF bound and unbound sensor configuration, S reports on the molecular stoichiometry. The population with stoichiometry around S ~ 0.55 (mid S, $H1^G$ colocalized with $H2^R$, Fig. 2a) contains the TF bound to both half-sites and the population with S ~ 1 represents the unbound biosensor (high S, $H1^G$ only). An increase in lacR bound biosensor with increasing lacR concentration is thus indicated by a gain in the relative area of the mid S population. The mid S population is much broader than the high S population due to the contribution of two fluorescent signals and because of additional intensity variations of the red dye Atto647N [11,12]. The fraction of TF bound DNAs is determined by estimating the mixing proportion of the mid S population using a bimodal fit model (Fig. 2a,b).

2.2. Improved model for stoichiometry distributions

An important aspect of accurately quantifying TFs, i.e. measuring the fraction of colocalized half-sites, is the use of a probability distribution function (PDF) that describes the two populations' (bound and unbound biosensors) best. Therefore, we carefully examined different PDFs and parameter estimation approaches. We tested maximum likelihood estimation(MLE) and non-linear least-squares (NLS) parameter estimation with different weights and compared Gaussian and three parameter Student's t distributions (see Materials and methods and Supporting Information) [13,14]. It should be noted that the Student's t distribution comprises the Gaussian distribution as a limiting case and was used before to achieve robust modeling of biological data sets [15,16]. We combined data from ~20 movies of different fields of view in one histogram with appropriate binning to assess the TF bound fraction for one experiment. The histogram was then modeled using the approaches described above. Fit quality was judged by examination of the residuals and by calculation of Pearson's $\chi^2_{\text{red}} = \frac{1}{\text{d. o. f.}} \sum_i \frac{(O_i - E_i)^2}{E_i}$ (red = reduced, d.o.f. = number of degrees of freedom, O_i are the observed counts and E_i are the expected counts). In addition, we performed a χ^2-goodness-of-fit test with the null hypothesis that the data are a random sample from the respective PDF (see Supporting Information) [13,14].

Both NLS estimation with weights equal to the inverse of the expected number of bin entries and MLE using a linear combination of two Student's t-distributions performed best [13]. We selected the NLS estimation to evaluate all the data due to computational speed. A comparison of the chosen fitting approach with the commonly applied ordinary non-linear least-squares (ONLS) estimation using a linear combination of two Gaussians (Fig. 2b) illustrates that the latter misses the tails of the narrow high S population. Only in the case of the Student's t-distributions χ^2_{red} is close to one and the null hypothesis of the χ^2-goodness-of-fit test cannot be rejected at the 5% significance level (see Supporting Information). Fig. 2c compares the Gaussian with the Student's t model based on the resulting relative deviation of the DNA colocalization. It is evident that the Gaussian model shows a systematic positive bias of up to 200% with respect to the Student's t model. In more than 50% of the experiments the TF bound fraction is overestimated by at least 10–20%. We also found that the bias becomes more significant for low TF concentrations (see Supporting Information Fig. S1).

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