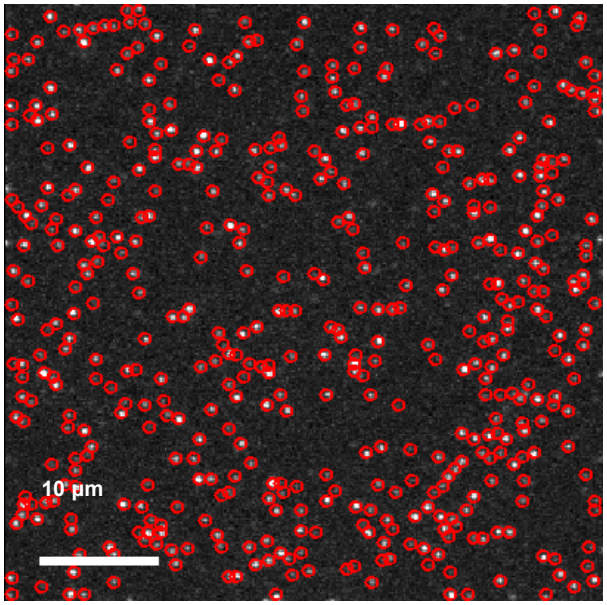
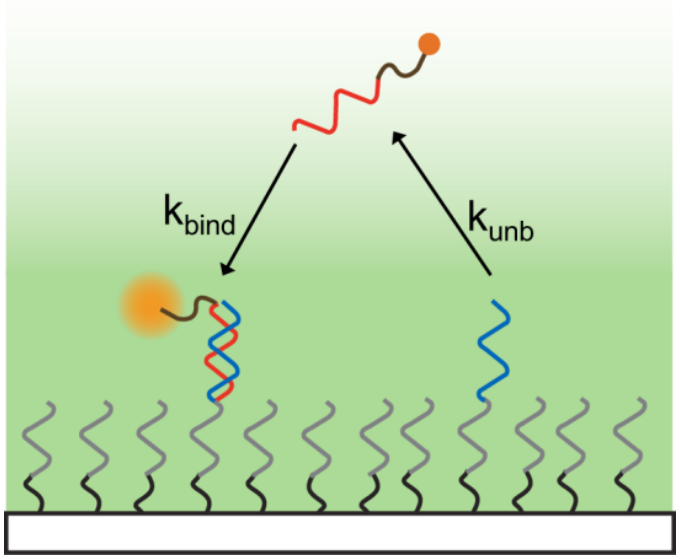
Single-molecule Fluorescence Microscopy: Quantitative Analysis by Counting Individual Molecules

Joel M. Harris, University of Utah



Quantitative analysis by imaging and counting molecules

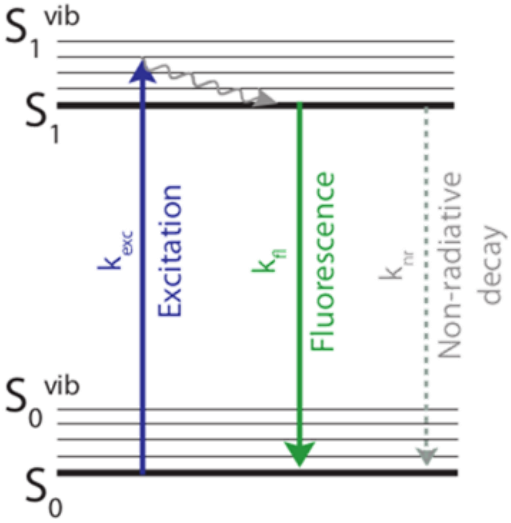
Measuring single-molecule DNA hybridization kinetics

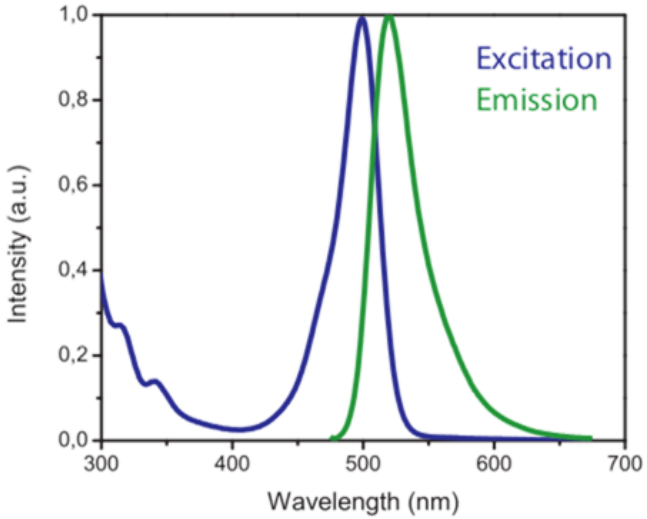


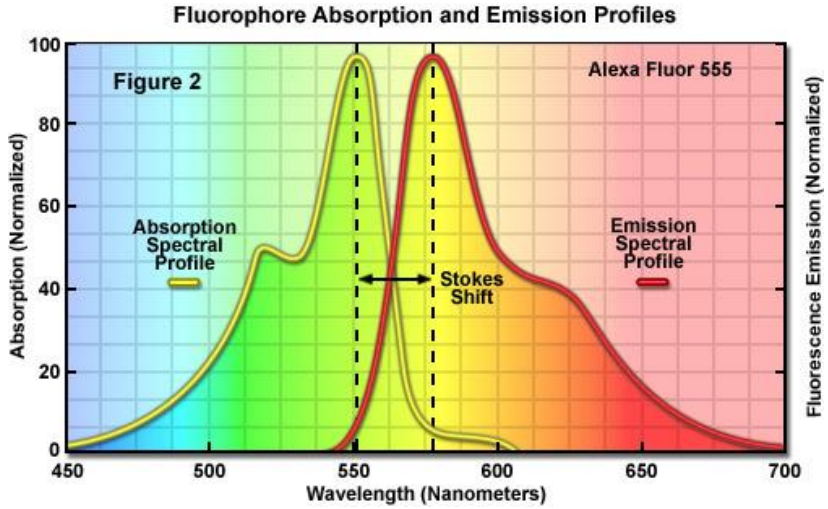
Sensitive detection of molecules by excitation of fluorescence

excited electronic state

ground state



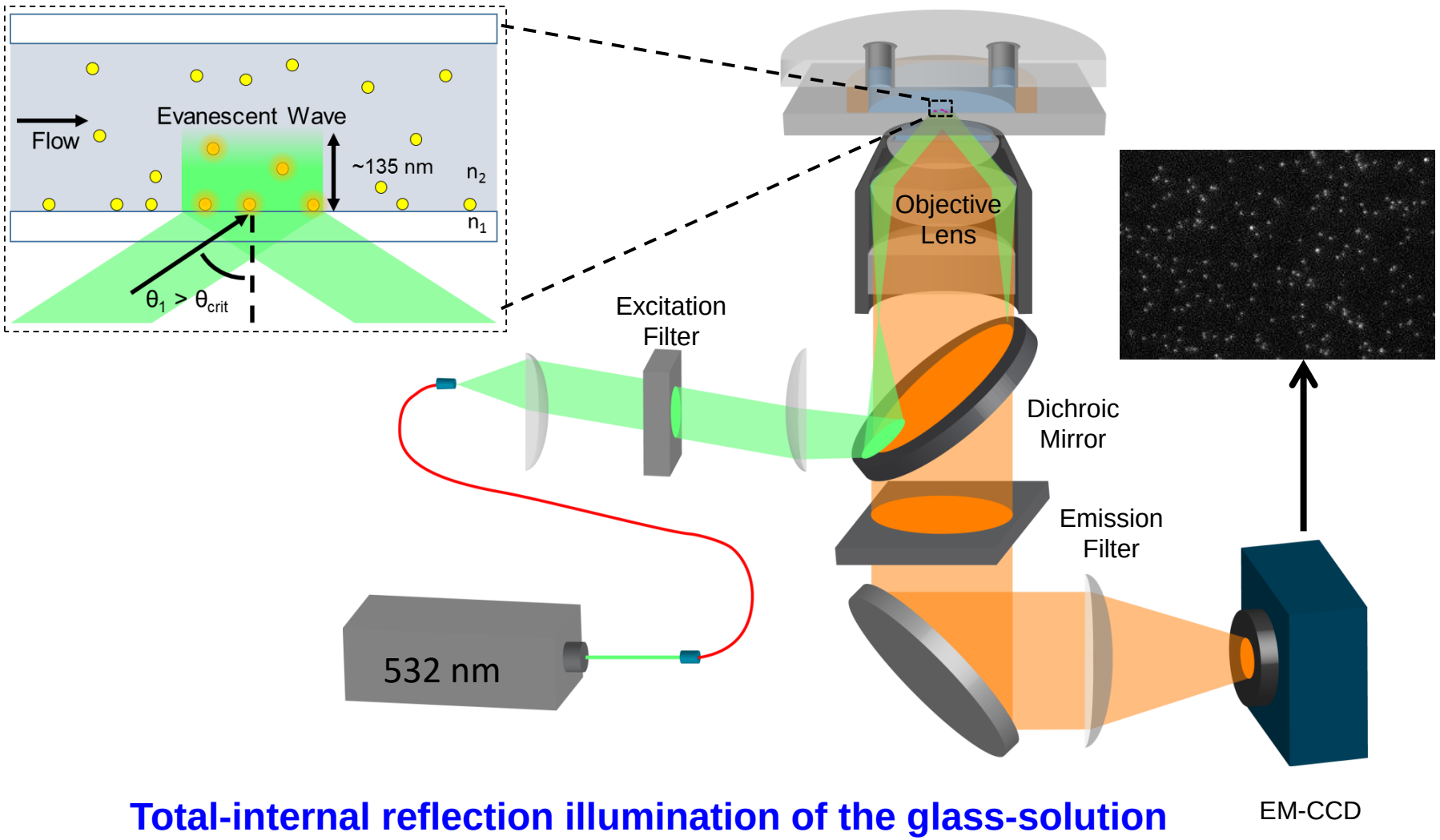




https://svi.nl/Fluorescence

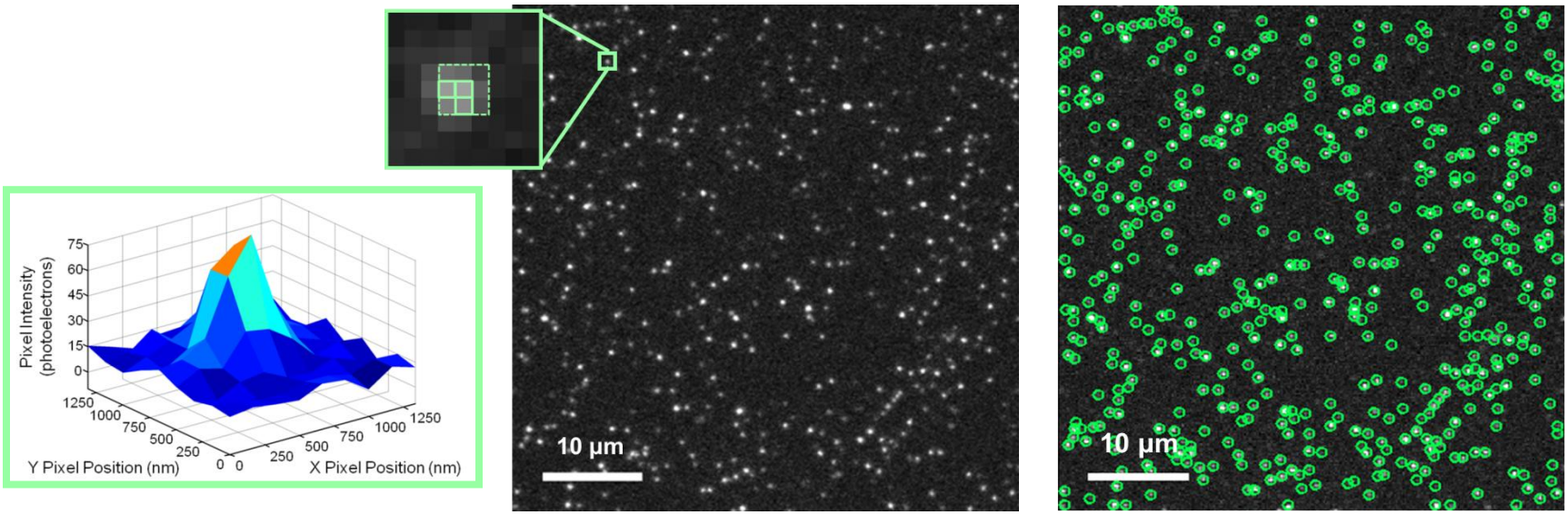
http://zeiss-campus.magnet.fsu.edu/articles/basics/fluorescence.html

Imaging of molecules on surfaces by total-internal reflection fluorescence microscopy



Total-internal reflection illumination of the glass-solution interface. Image molecules that accumulate in the evanescent wave depth (~135 nm).

Quantitative analysis of interfacial populations by identifying and counting individual molecules



Counting of single fluorescent molecules on surfaces using *spatial criteria*

Eric M. Peterson and Joel M. Harris, *Analytical Chemistry,* 82, 189 (2010).

False positives, α < 2.8 spots per frame; false negatives, β < 1.5% for single fluors.

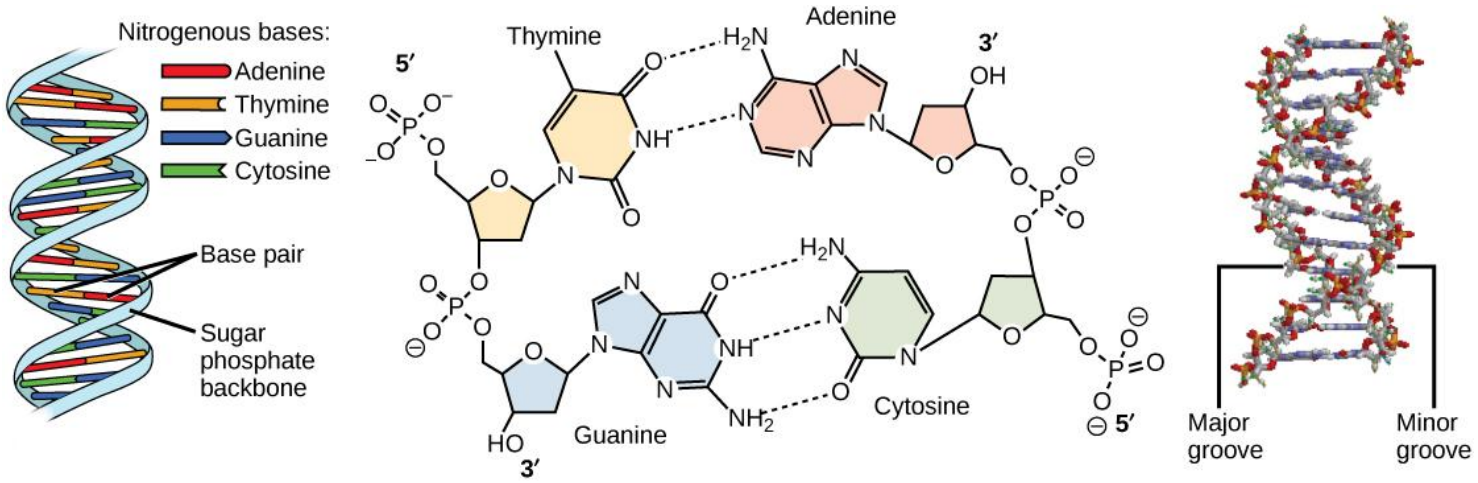
Studies of binding equilibria *at very low surface coverage*

Surface populations determined *without intensity calibration*

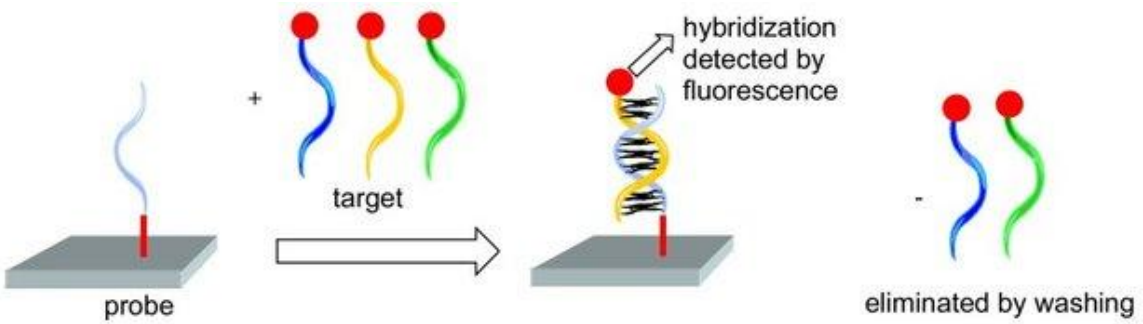
Observe interfacial kinetics *at equilibrium*

DNA duplex formation: detecting specific gene sequences

DNA base-pair recognition



Capturing specific DNA sequences with immobilized ‘probe’ strands



http://cnx.org/contents/GFy_h8cu@10.53:rZudN6XP@2/Introduction

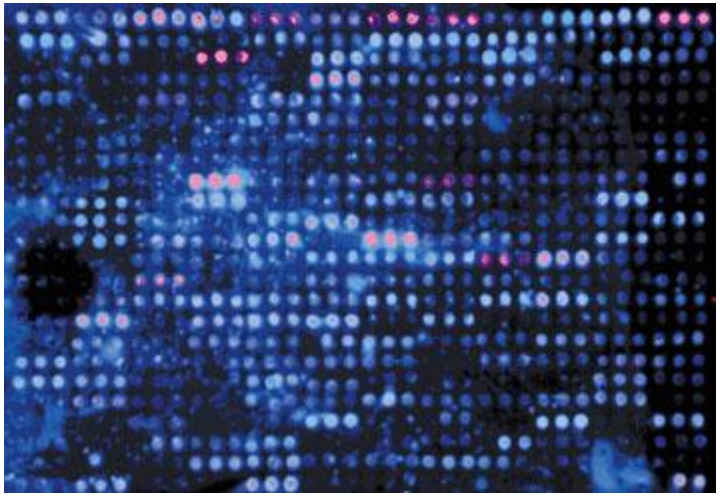
Sensors 2006, 6(8), 901-914; https://doi.org/10.3390/s6080901

DNA arrays for genetic disease detection

DNA microarrays are used in both research and medical diagnostics: Affymetrix GeneChip®

Understanding of affinity constants and kinetics can help with sensor design and operation.

Ultrasensitive (single-molecule) DNA analysis without amplification

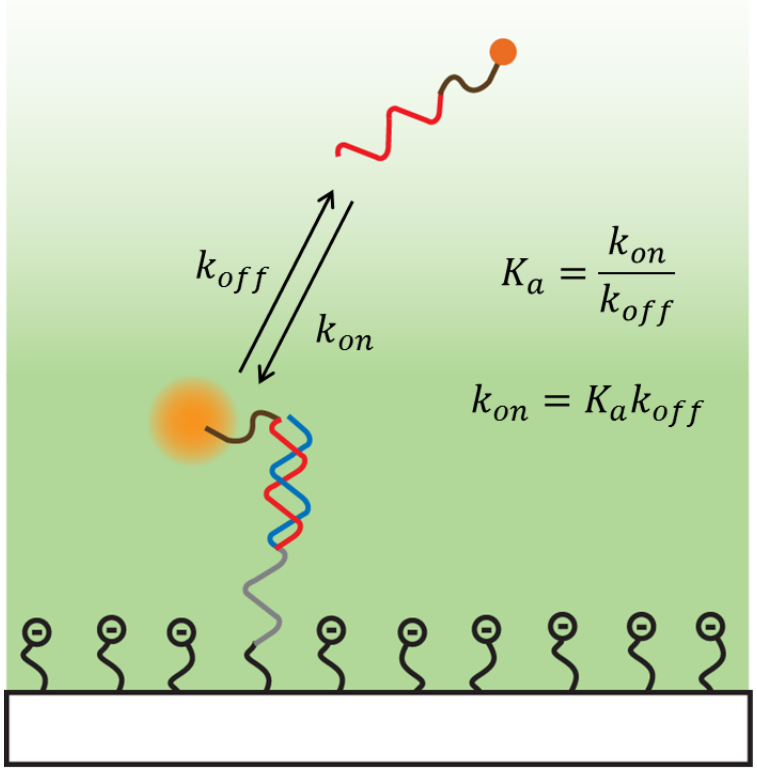


Dalma-Weiszhausz, D. D.; Warrington, J.; Tanimoto, E. Y.; Miyada, C. G. *Methods in Enzymology,* **2006**, *410*, 3 - 28.

Imaging single-molecule DNA hybridization

GOALS:

- **Immobilized DNA on passivated surfaces**
- **Quantify probe DNA binding site density and association constant**
- **Measure hybridization kinetics *at equilibrium***
- **Investigate the influence of ionic strength on DNA hybridization**
- **Single-molecule microarrays: study the influence of labeling on DNA hybridization, competitive assays**

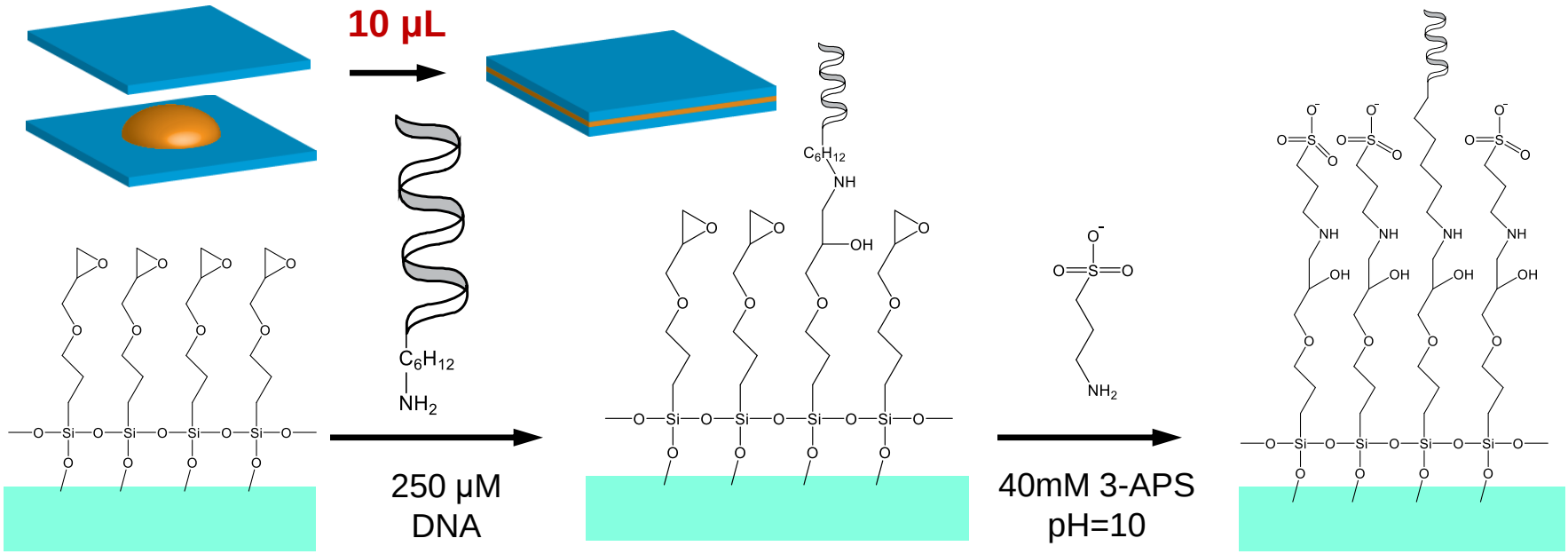


Design constraints: Capture substrates that can be observed for long periods of time (good statistics, probe site imaging).

Selectivity derived from a well-passivated surface.

Immobilizing amine-terminated DNA on an epoxide surface

DNA immobilization: **epoxide-coupling reaction with high amine-DNA concentration, followed by 3-aminopropanesulfonate passivation.**



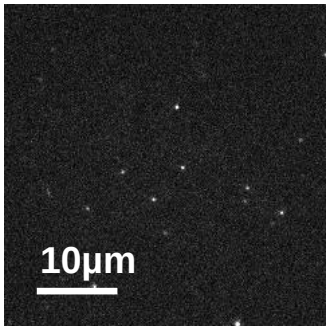
Testing DNA probe selectivity

Probe ssDNA: 5’- NH_2-GT CGG TAT ATC CCA T-3’

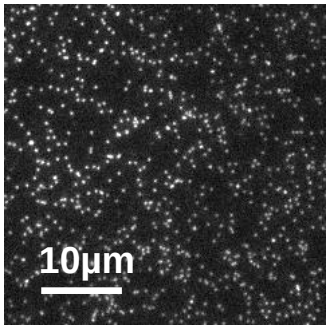
10-mer Target ssDNA: 3’-ATA TAG GGT A-5’-PEG_6-Cy3

10-mer Control ssDNA: 3’-TAG ATG ATG A-5’-PEG_6-Cy3

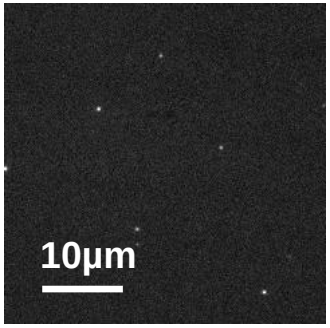
Sulfonate Surface
500-pM Target

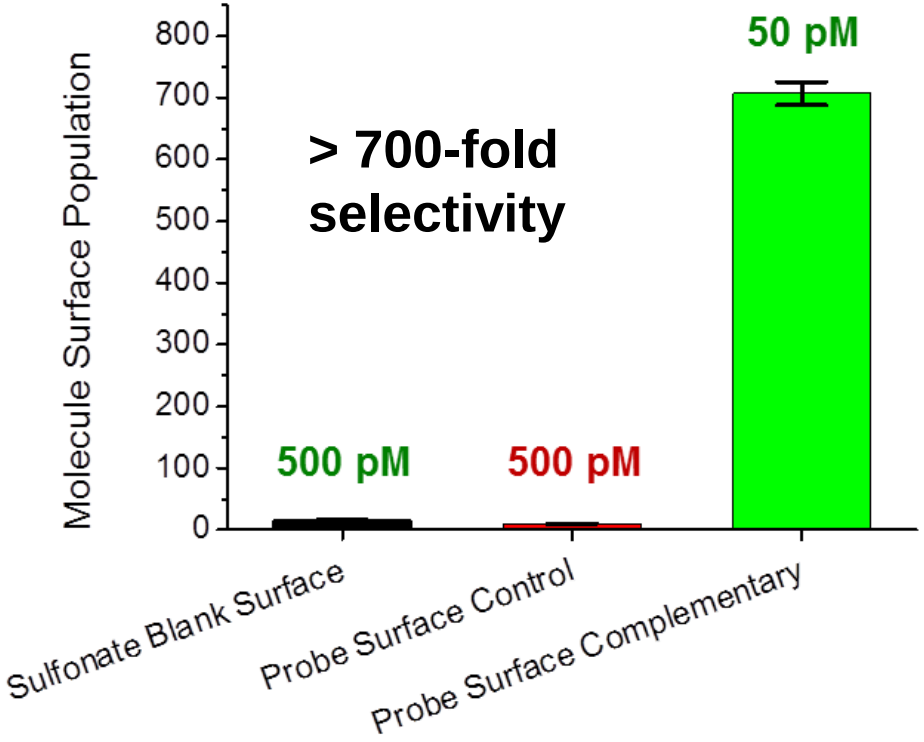


Probe Surface
50-pM Target

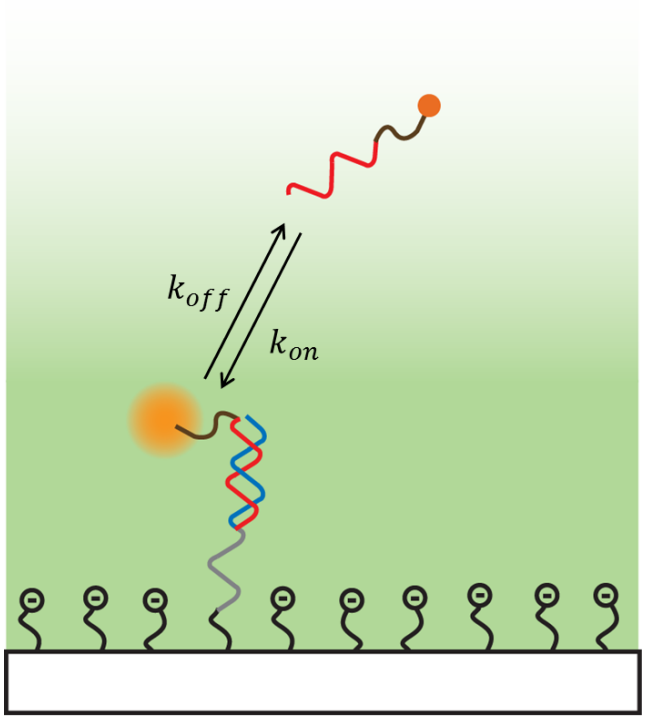


Probe Surface
500 pM Control

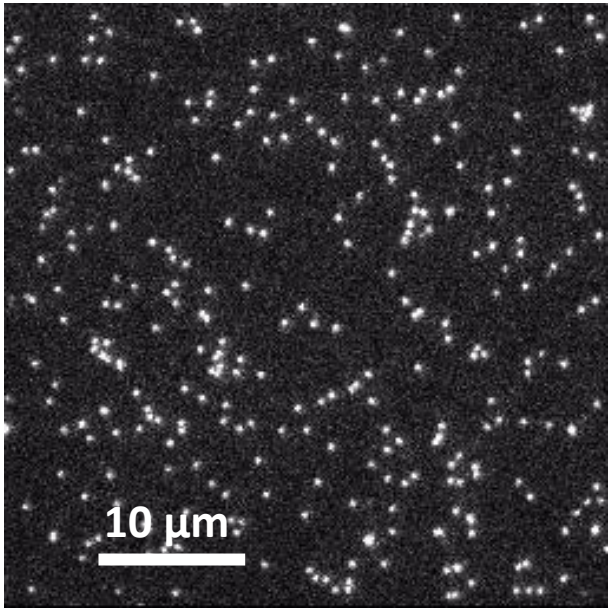




10-mer hybridization events are *reversible*



Hybridization at interface with immobilized probe



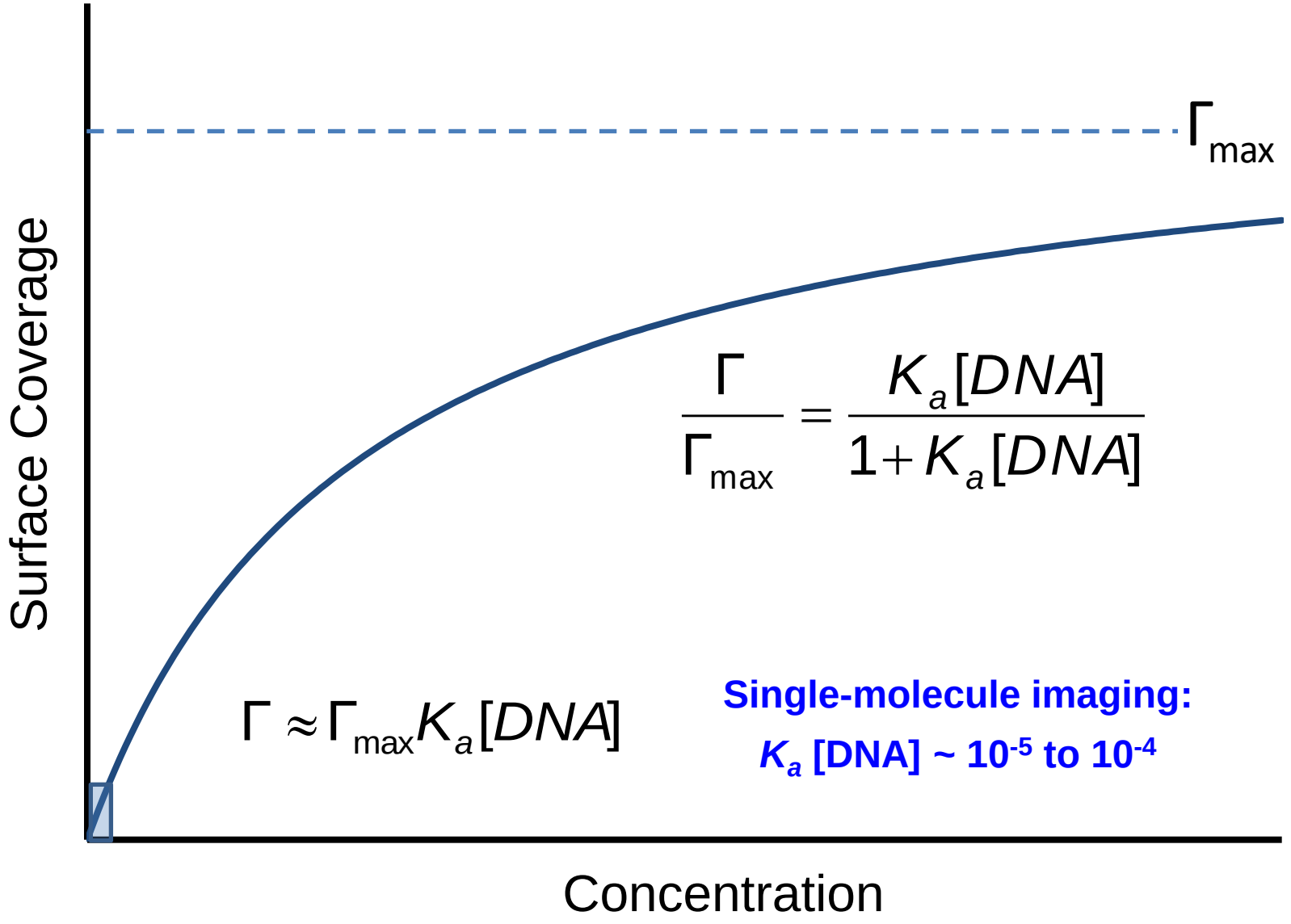
- Complementary DNA hybridization reversible near melting temperature
- Association and dissociation kinetics control population of labeled duplexes

Imaging at 0.5 Hz
Playback at 20x speed

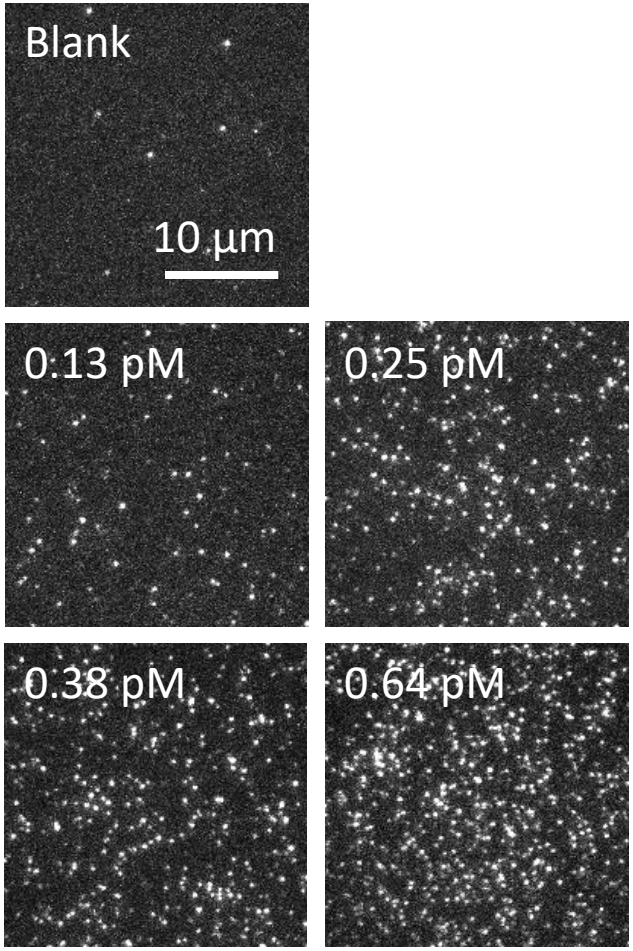
Jungmann et al. *Nano Lett.* **2010**, *10,* 4756

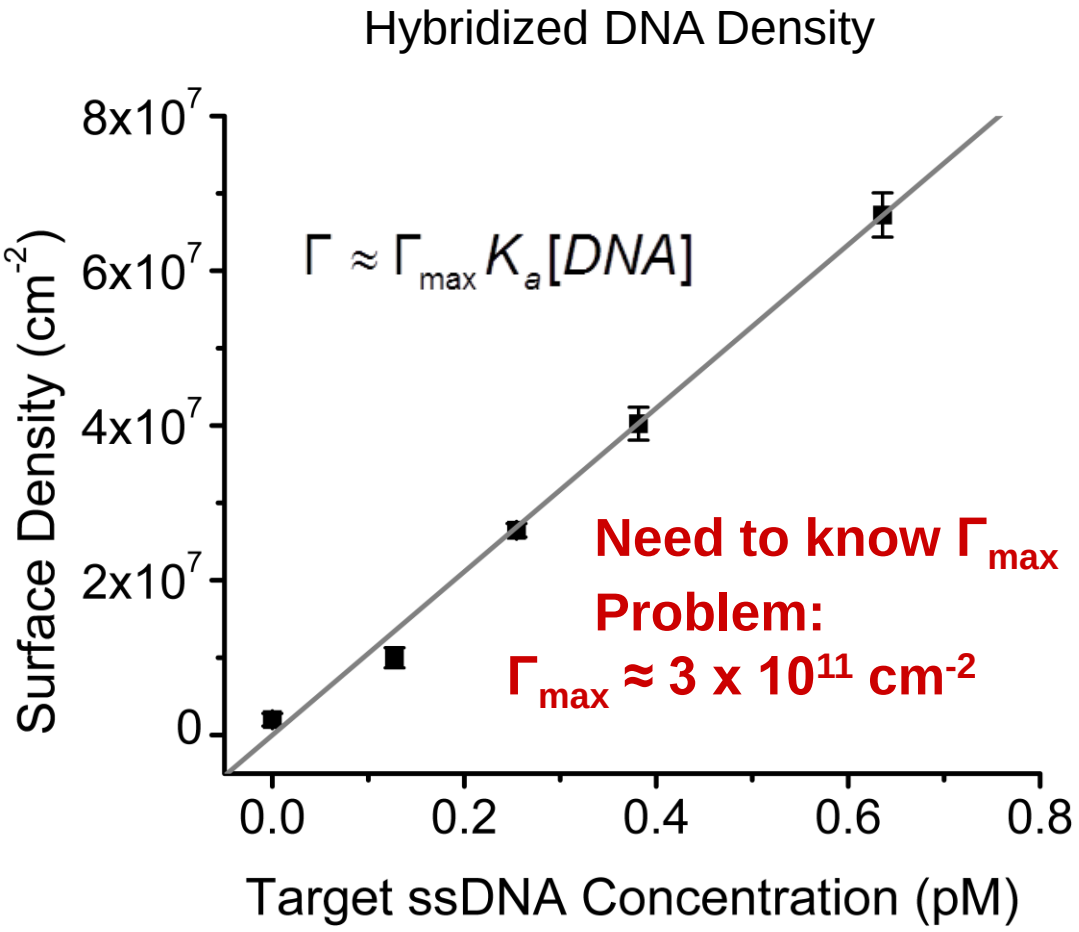
Chen, J.; Bremauntz, A.; Kisley, L.; Shuang, B.; Landes, C. F. *ACS Appl. Mater. Interfaces*, **2013**, *5*, 9338

Expected concentration response: Langmuir site-binding model

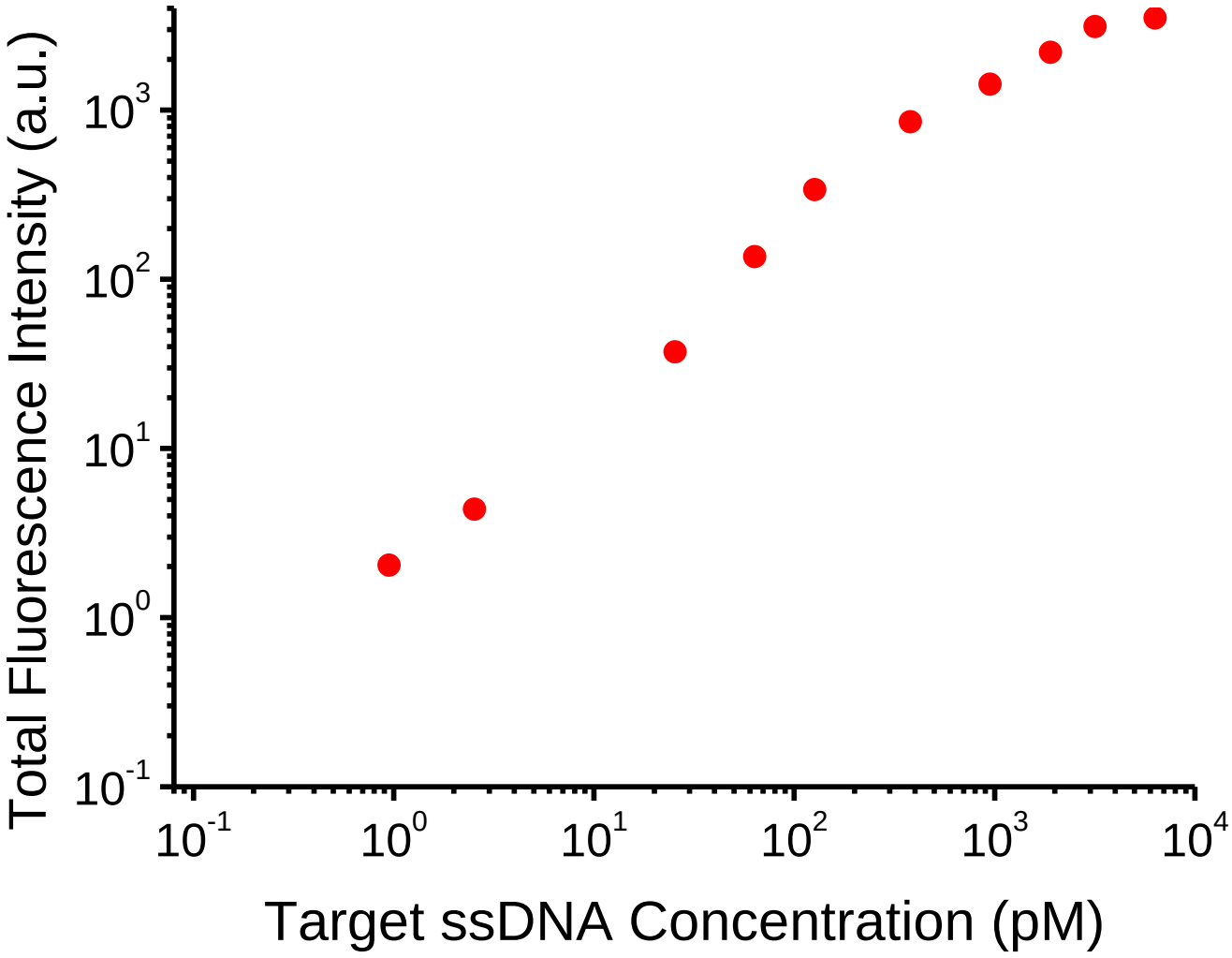


Target DNA concentration response



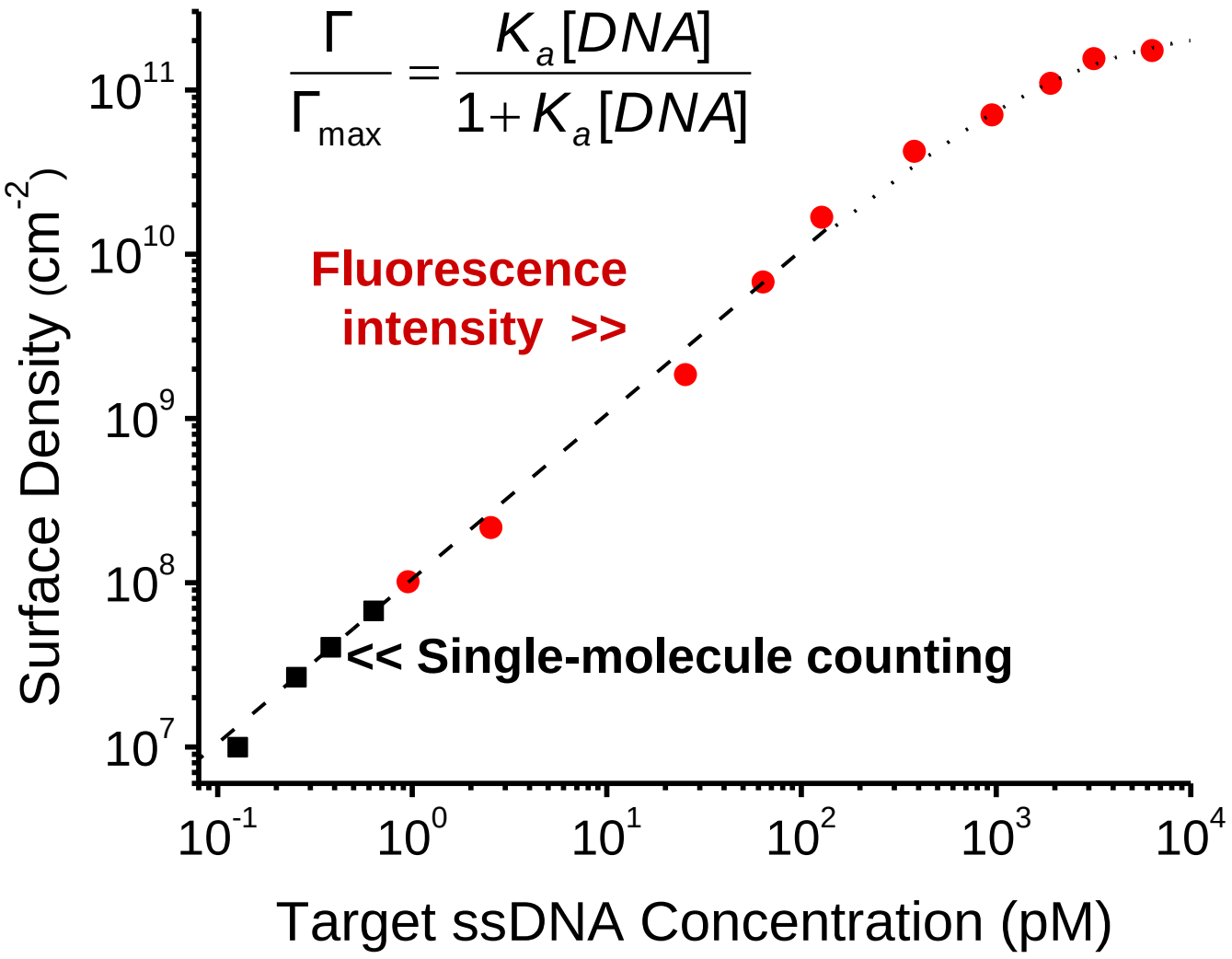


Total fluorescence intensity shows probe-site saturation

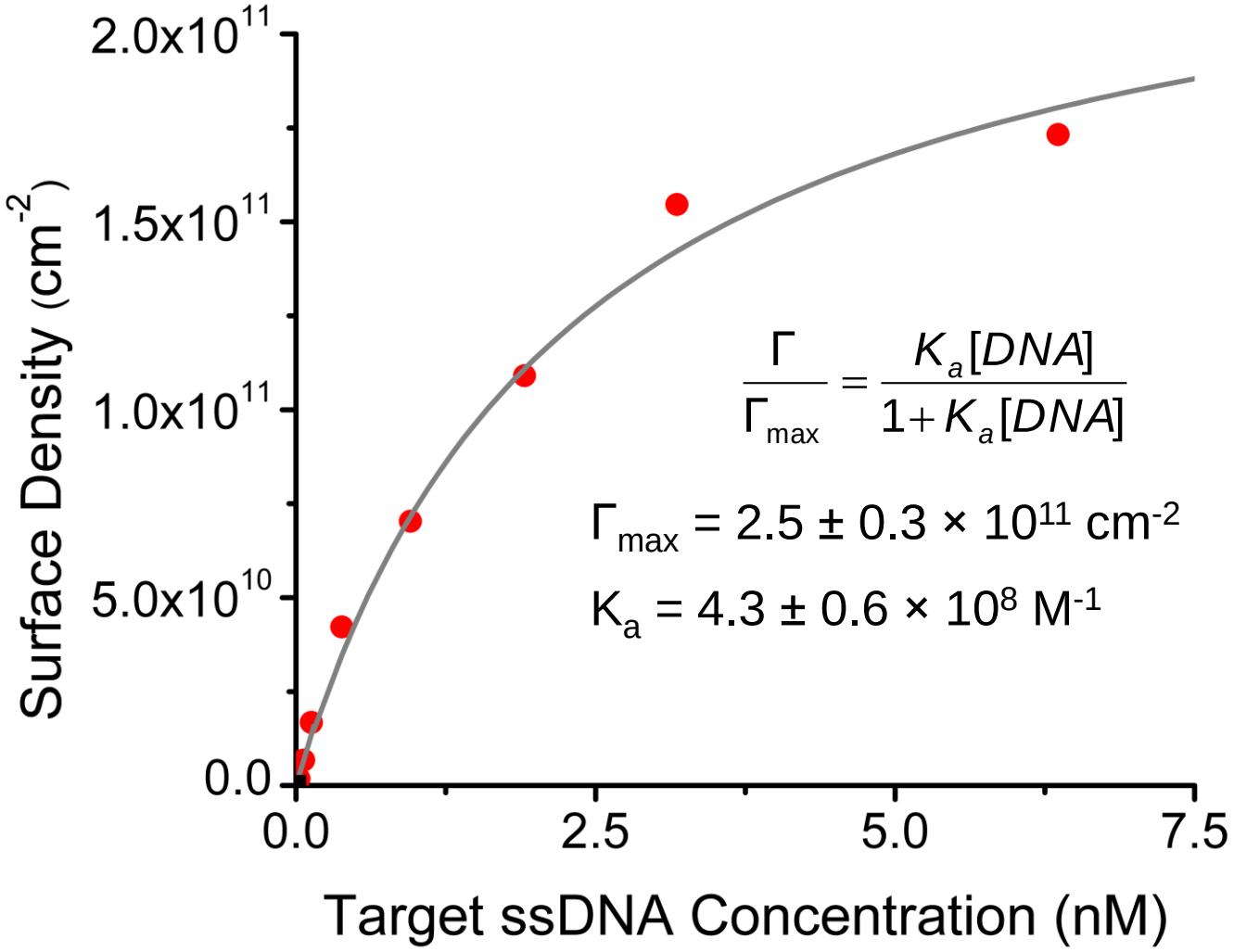


But the response is not calibrated!

Calibrating fluorescence intensities with *single-molecule standards*

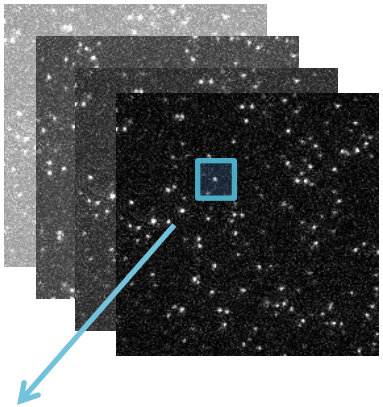


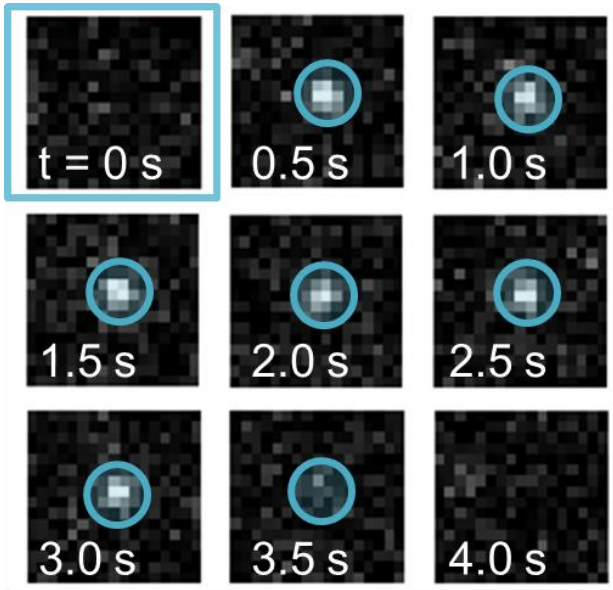
Calibrated fluorescence intensity isotherm

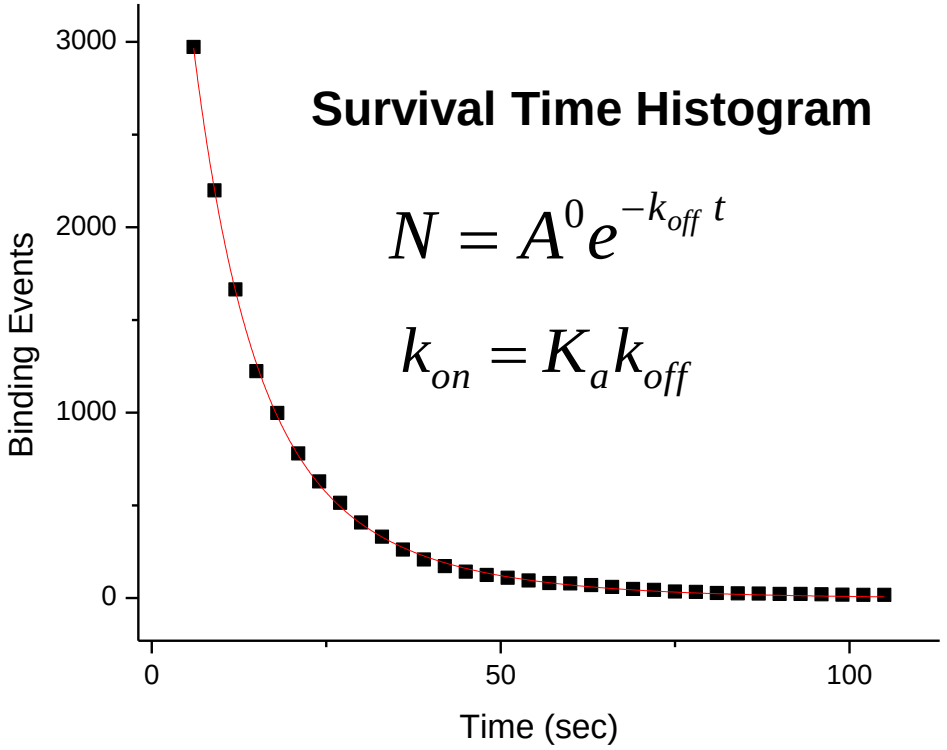


Obtain probe density and duplex association constant

Measuring duplex dissociation rates at equilibrium



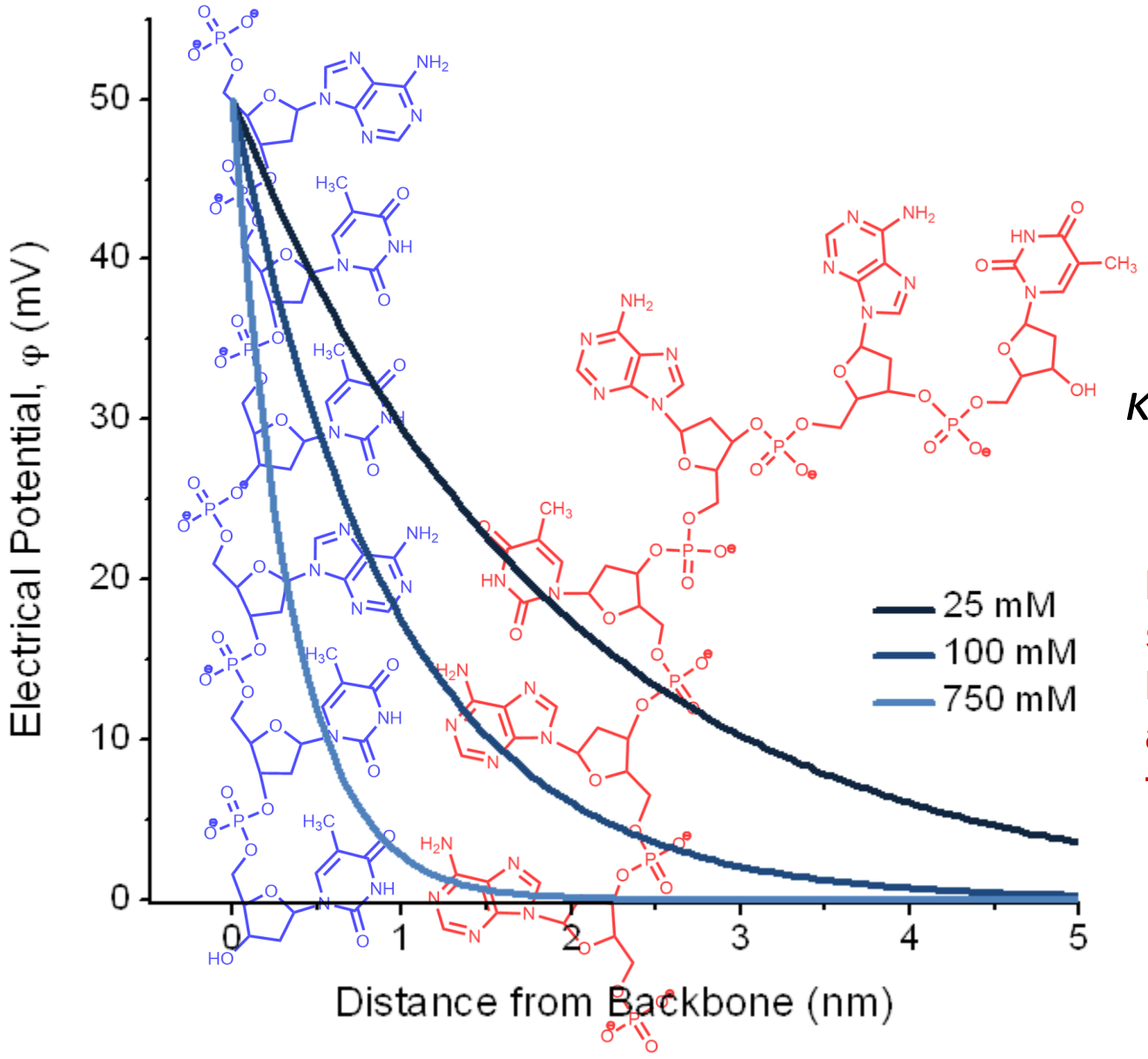




10-mer duplex	K_a (μM^{-1})	k_{on} x 10^{-6} (M^{-1} s^{-1})	k_{off} (s^{-1})
Immobilized	38 ± 1	1.64 ± 0.06	0.043 ± 0.001
Free-Solution	31 ± 2	1.41 ± 0.08	0.046 ± 0.004

Immobilized DNA exhibits comparable association constants and hybridization kinetics as in free solution: *Anal. Chem.* 88, 1345 (2016).

Electrostatic interactions between complementary strands

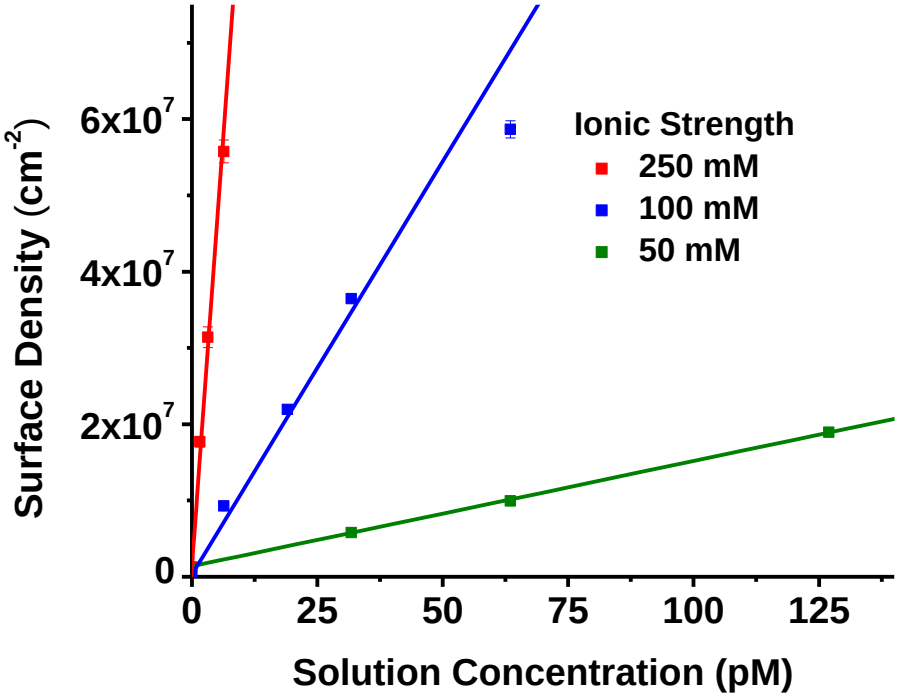


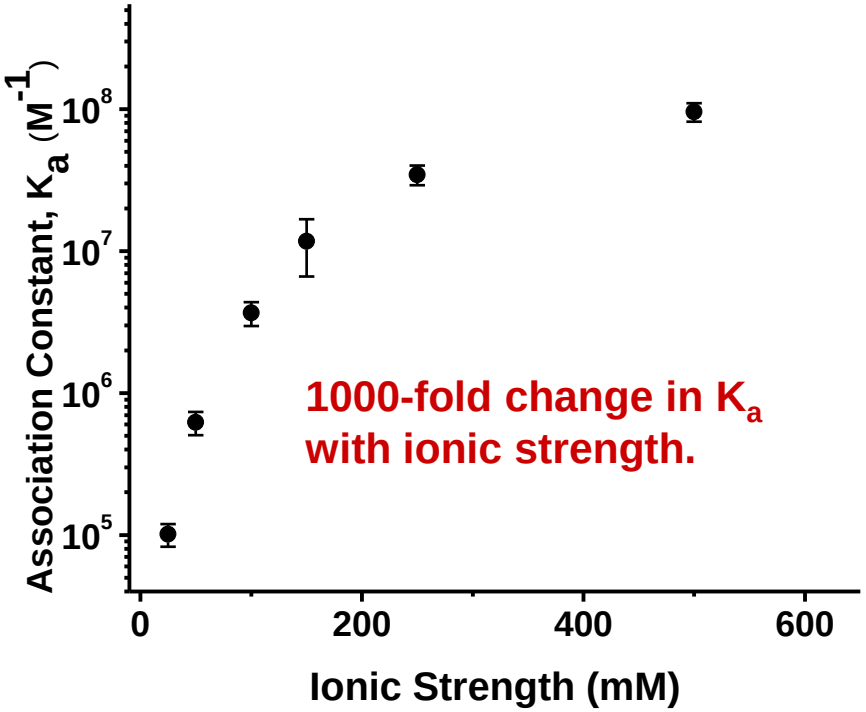
$$\varphi(x) \approx \zeta e^{-x/\kappa^{-1}}$$

$$\kappa^{-1} = \left(\frac{\varepsilon \varepsilon_0 kT}{2C^0 z^2 e^2} \right)^{1/2}$$

Ionic strength should influence DNA duplex stability and the binding transition state.

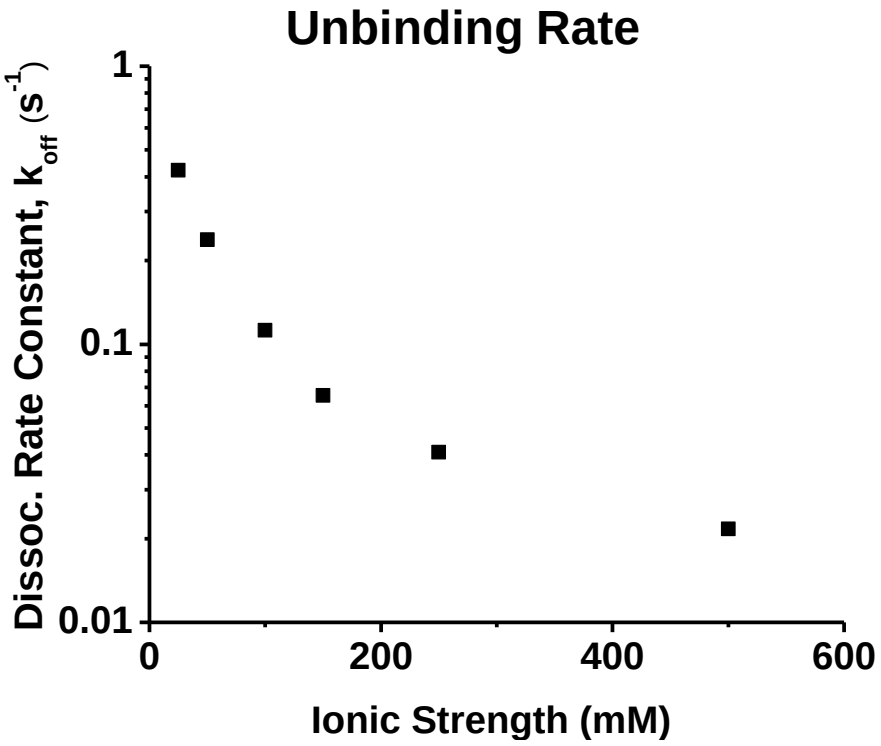
Ionic strength strongly governs the association constant

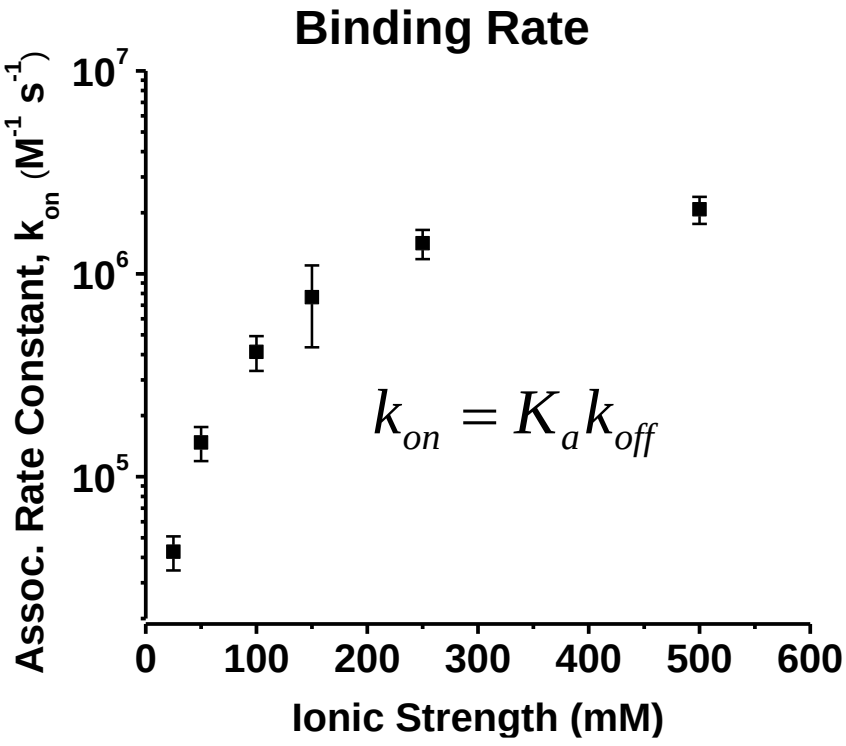




How about the kinetics of DNA hybridization?

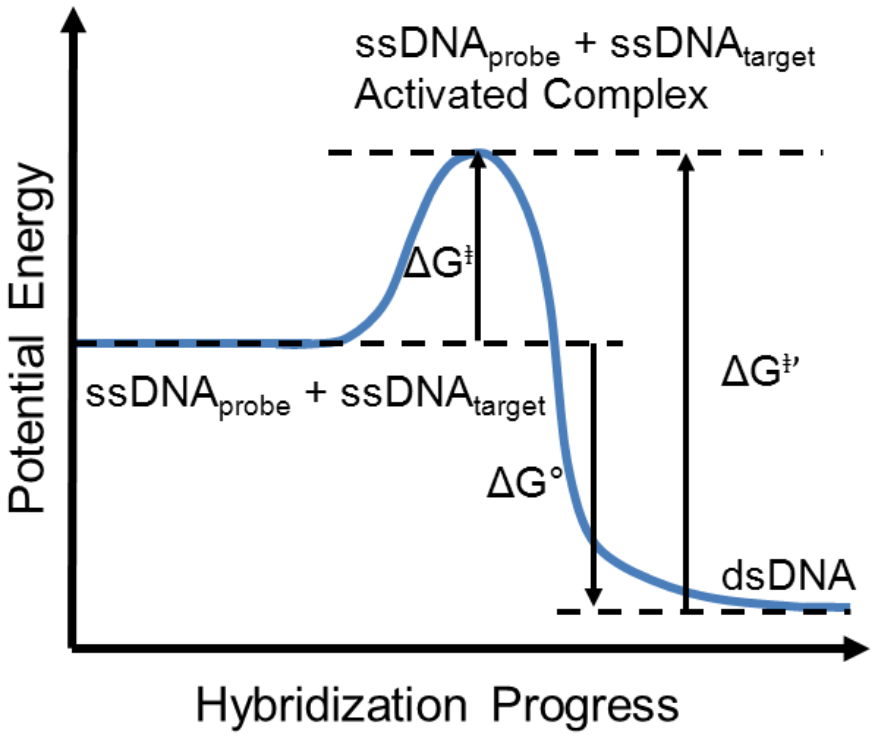
Both rate constants are very sensitive to ionic strength





Can we model the electrostatic interactions that influence DNA hybridization kinetics?

Modeling electrostatic contributions to DNA binding energies

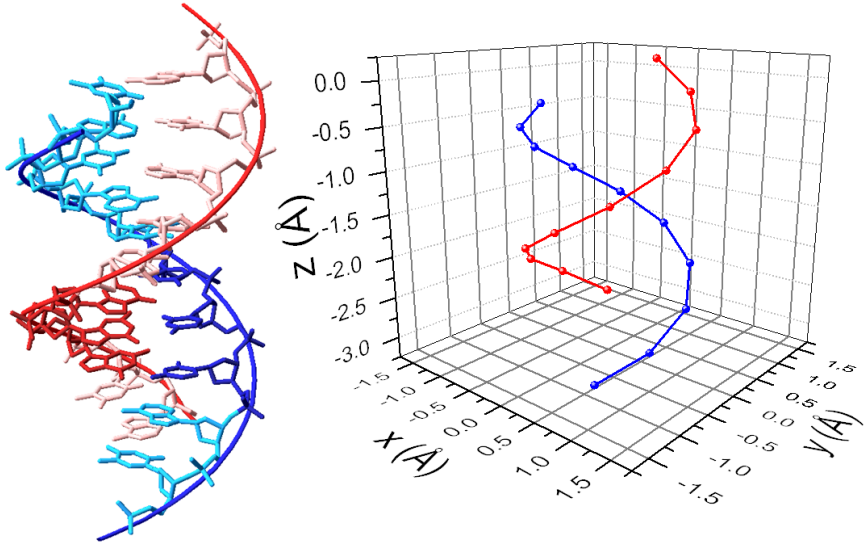


$$\Delta G^\circ = -RT \ln (K_a) = \Delta G^\circ_{es} + \Delta G^\circ_{nes}$$

$$\Delta G^\ddagger = -RT \ln (k_{on}\, h/k_B T) = \Delta G^\ddagger_{es} + \Delta G^\ddagger_{nes}$$

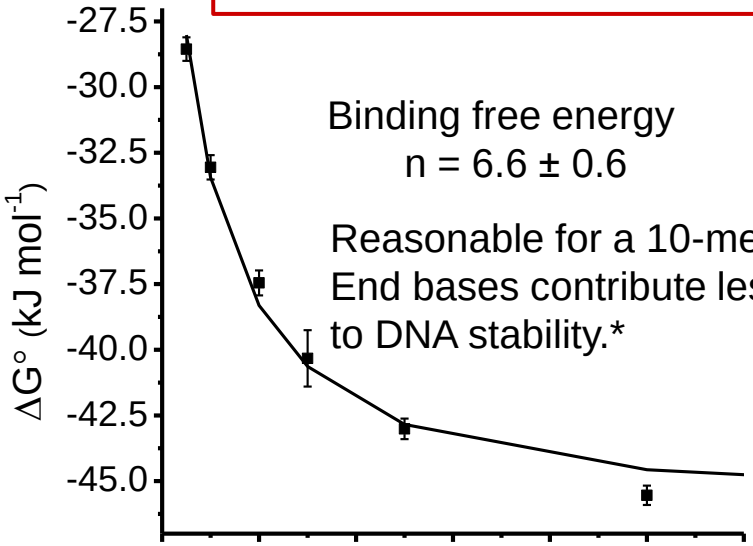
$$\Delta G_{es}(n) = \frac{N_A (ez)^2}{4\pi\varepsilon\varepsilon_0} \sum_{i=1}^{n} \sum_{j=1}^{n} \left[\frac{\exp(-\bar{r}_{ij}/\kappa^{-1})}{\bar{r}_{ij}} \right]$$

***Vary n*, number of interacting charges.**
r_{ij} from DNA structure.*



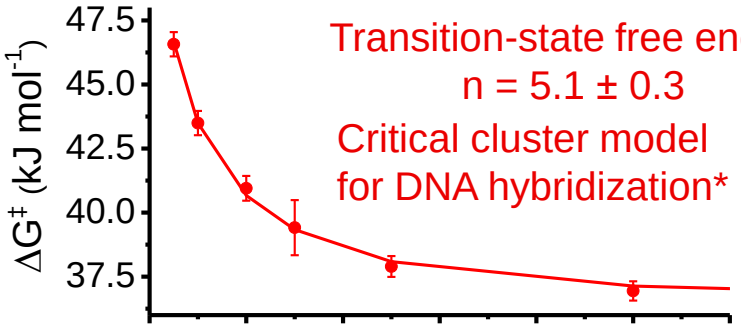
* Guohui Zheng, Xiang-Jun Lu, and Wilma K. Olson, Nucleic Acids Research, 2009, **37** (suppl 2), W240-W6.

Electrostatic contributions to DNA binding energies



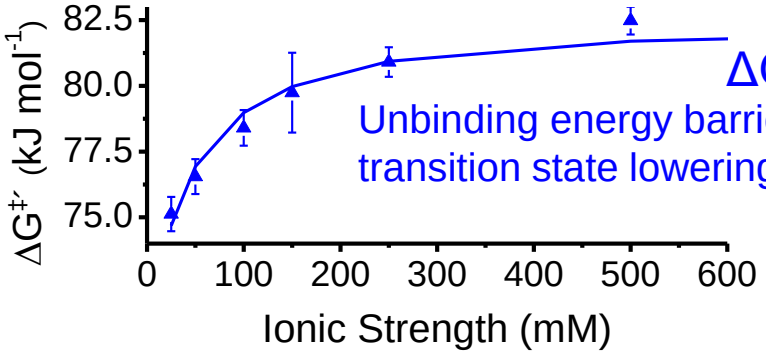
Binding free energy
n = 6.6 ± 0.6

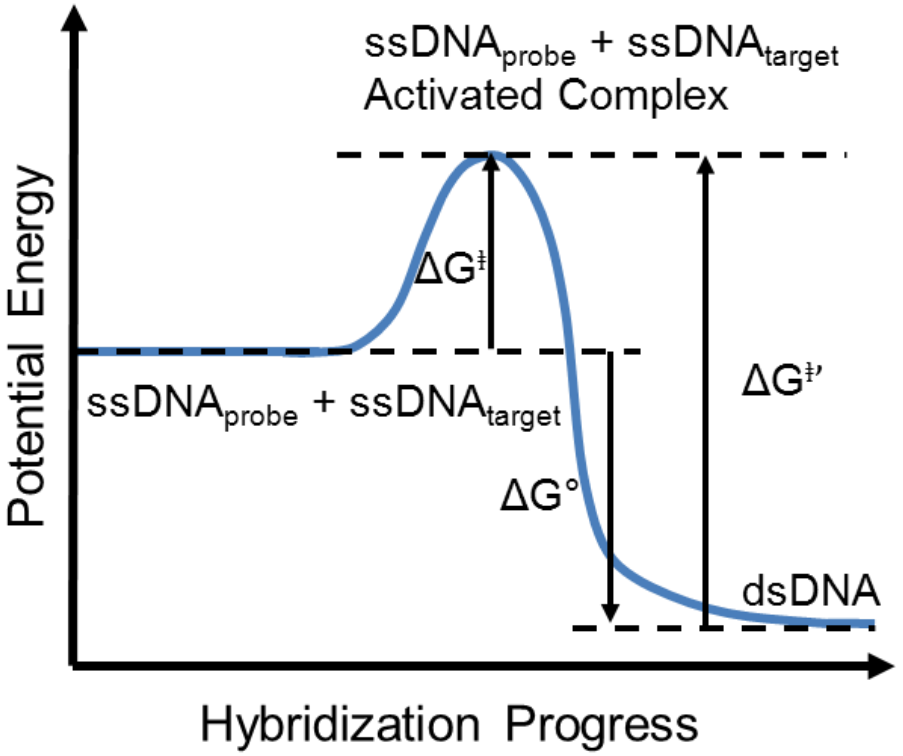
Reasonable for a 10-mer.
End bases contribute less
to DNA stability.*



Transition-state free energy
n = 5.1 ± 0.3

Critical cluster model
for DNA hybridization*



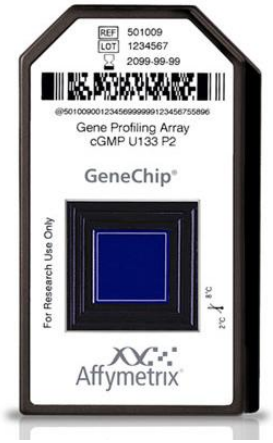


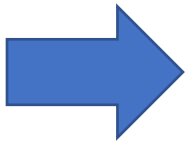
$\Delta G^{\ddagger\prime} = \Delta G^{\ddagger} - \Delta G^{\circ}$

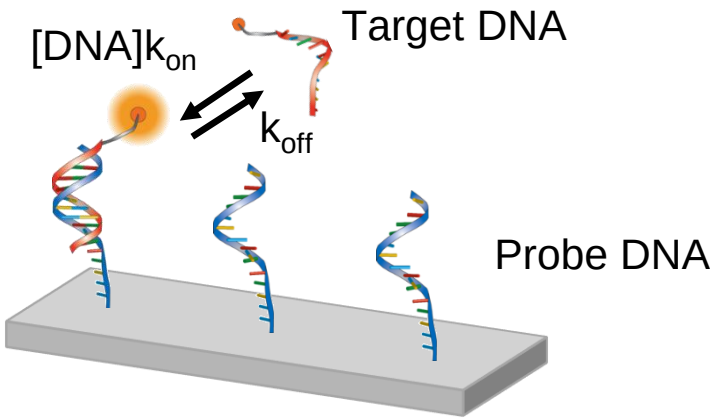
Unbinding energy barrier at higher ionic strength balances transition state lowering with greater duplex stability

*J. G. Wetmur, Annual Review of Biophysics and Bioengineering, 1976, **5**(1), 337-61.

Scaling DNA microarrays to the molecular limit: a spot is an individual DNA probe strand







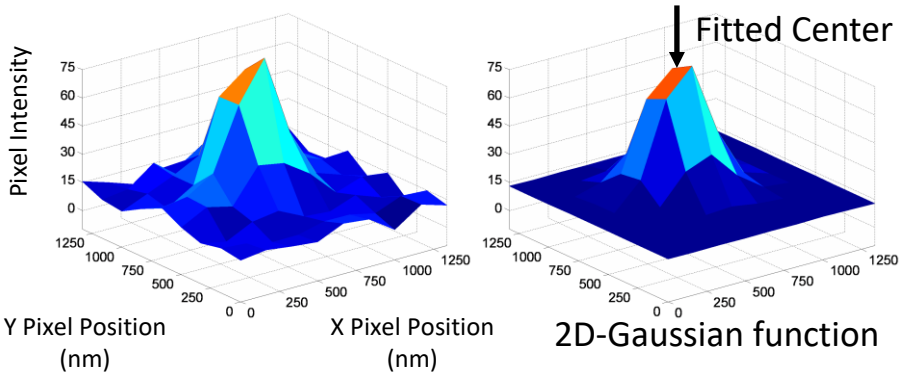
Dalma-Weiszhausz, D. D.; Warrington, J.; Tanimoto, E. Y.; Miyada, C. G. *Methods in Enzymology,* **2006**, *410*, 3

- **On- and off-rates from observing individual probe molecules**
- **Characterize heterogeneity on a per-molecule basis**
- **Identify individual probe DNA molecules on the surface**
- **Measure the kinetics of *unlabeled* target strands**

- **Technical challenges: track (unlabeled) probe sites by their locations. Need spatial resolution and stability.**

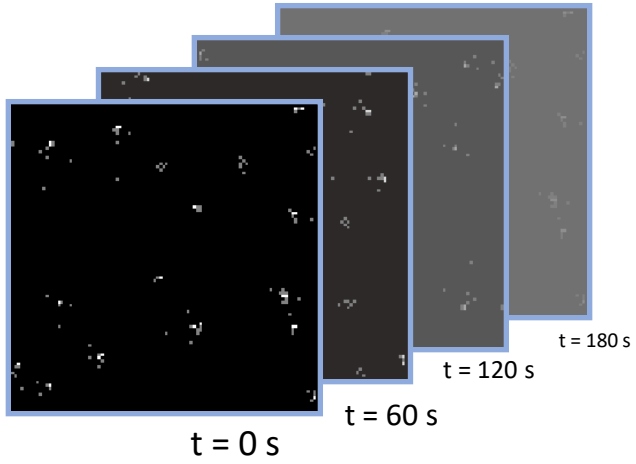
Locating probe DNA sites and correcting stage drift

1. Locate centroids with a fit to the PSF



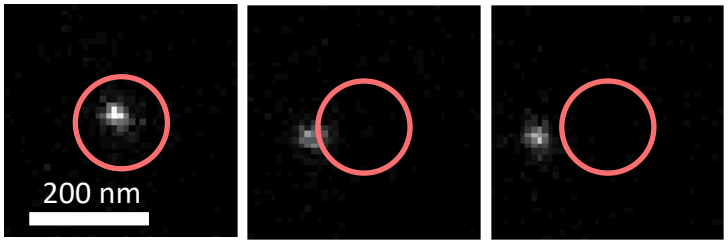
2. Generate high-resolution map of target coordinates in time

$\rho_t(\vec{\boldsymbol{R}}, t)$

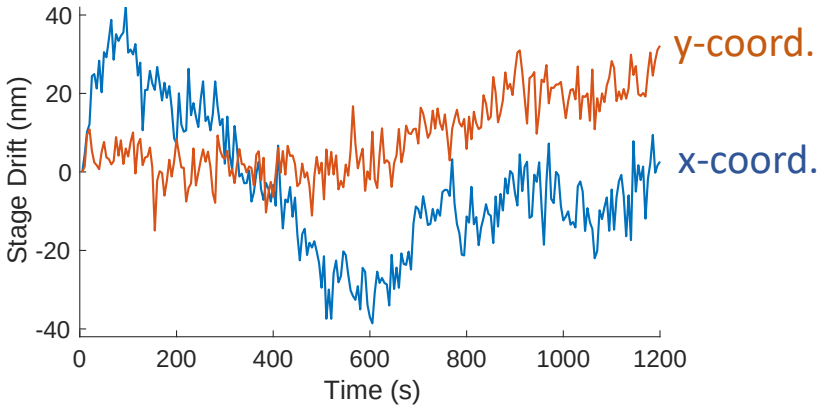


3. Measure stage drift with spatial-temporal autocorrelation

$$f(\vec{\boldsymbol{r}}, \tau) = \langle \rho_t(\vec{\boldsymbol{R}}, t)\rho_t(\vec{\boldsymbol{R}} - \vec{\boldsymbol{r}}, t + \tau) \rangle$$



4. Generate coordinate corrections (20-nm resolution) to compensate for stage drift.



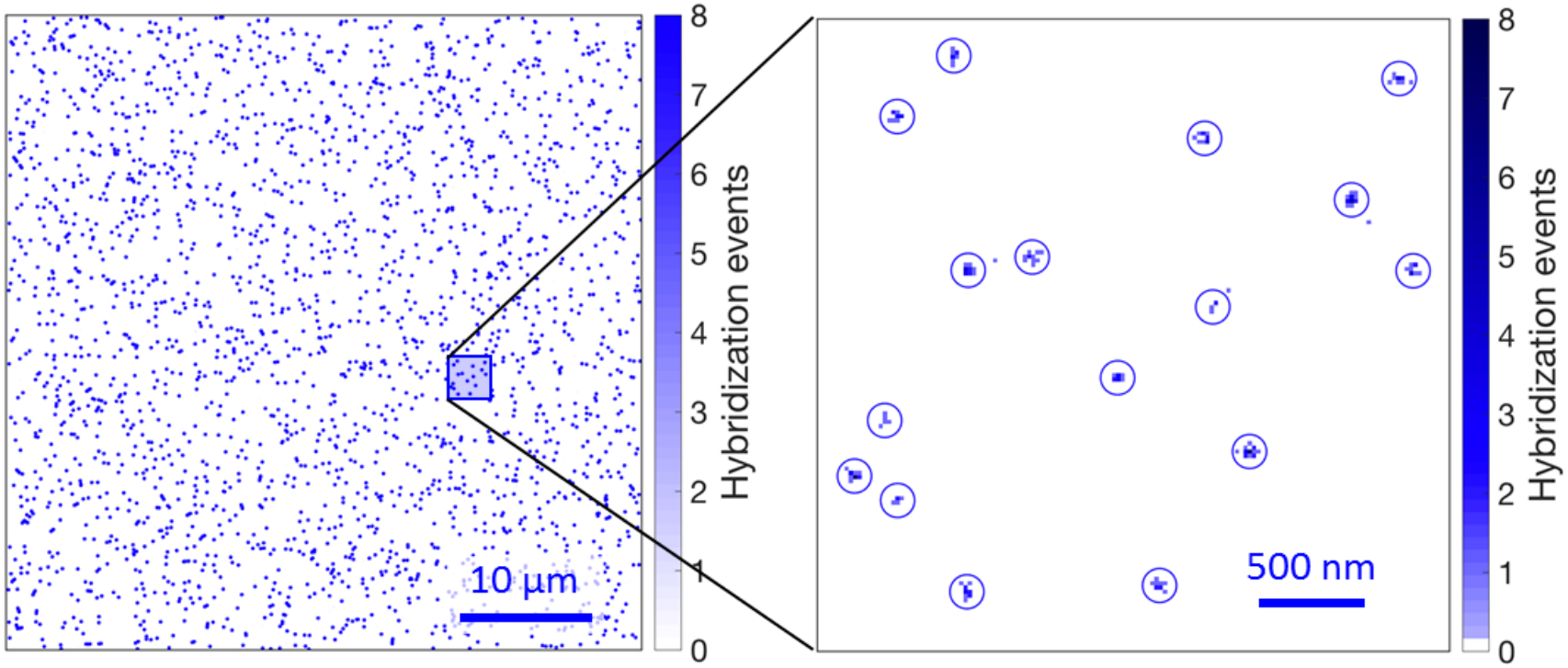
Super-resolution imaging of DNA hybridization

Mapping locations of reversible hybridization events reveals *individual immobilized probe molecules*:

Surf-NH-5'-GTC GGT ATA TCC CAT-3'

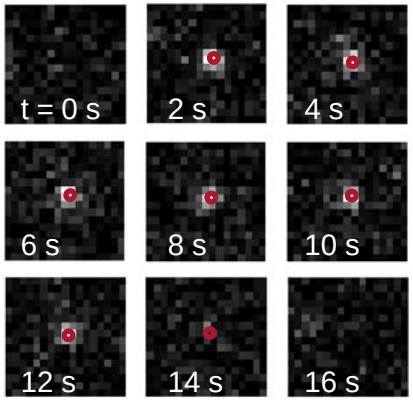
A TAT AGG GTA-5'-PEG$_6$-Cy3

Hybridization at 24.0 °C, 250 mM NaCl



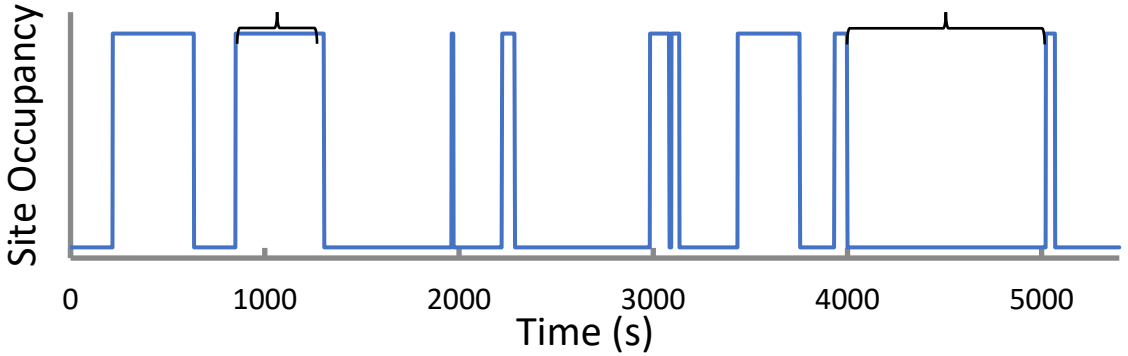
Measuring hybridization kinetics at individual probe DNA

Track each probe DNA over time

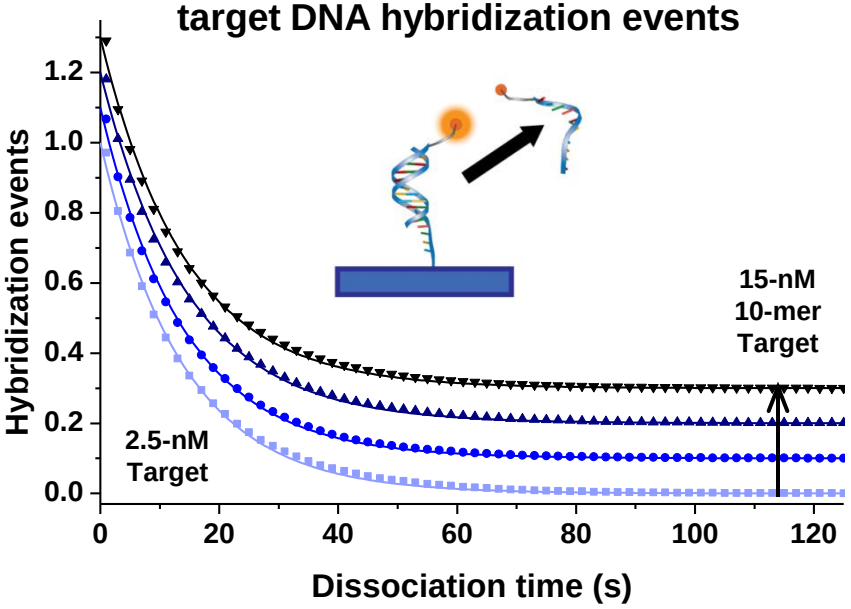


time to dissociate
$\tau_{off} = k_{off}^{-1}$

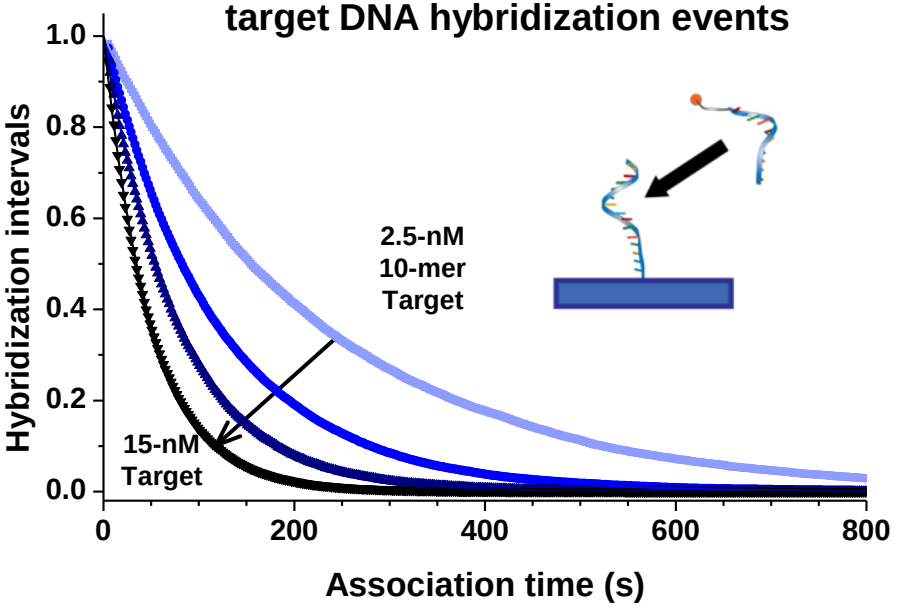
time to associate
$\tau_{on} = ([DNA]k_{on})^{-1}$



Dissociation time histogram of all target DNA hybridization events

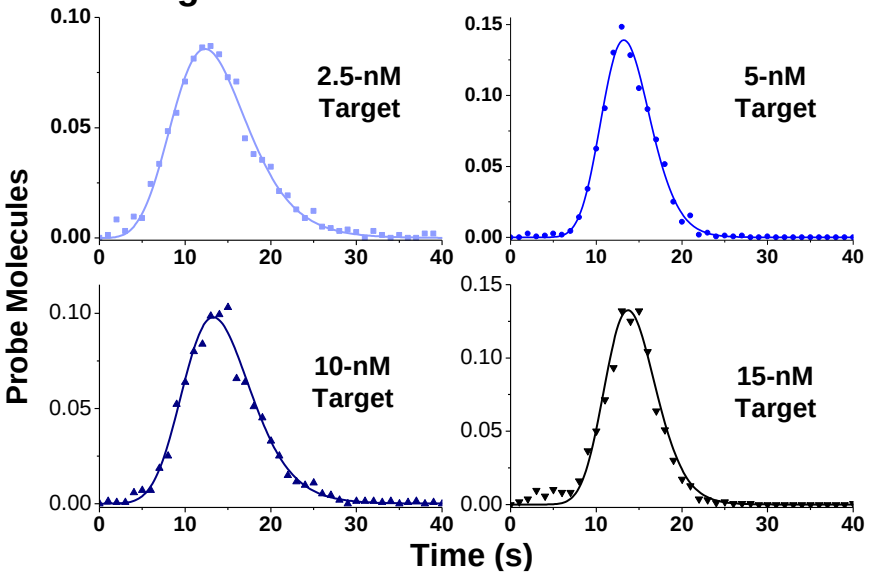


Association time histogram of all target DNA hybridization events

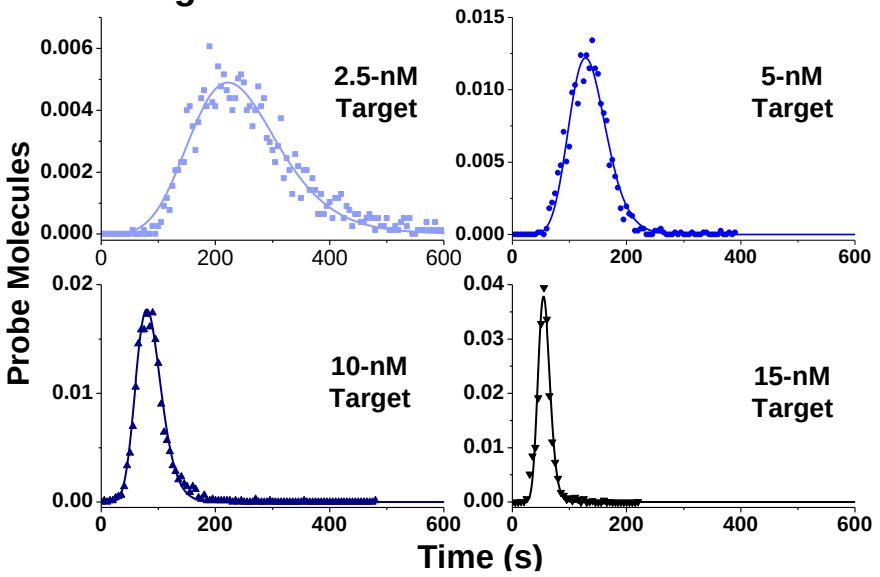


Hybridization kinetics of *individual* probe molecules

Average dissociation times of immobilized DNA



Average association times of immobilized DNA

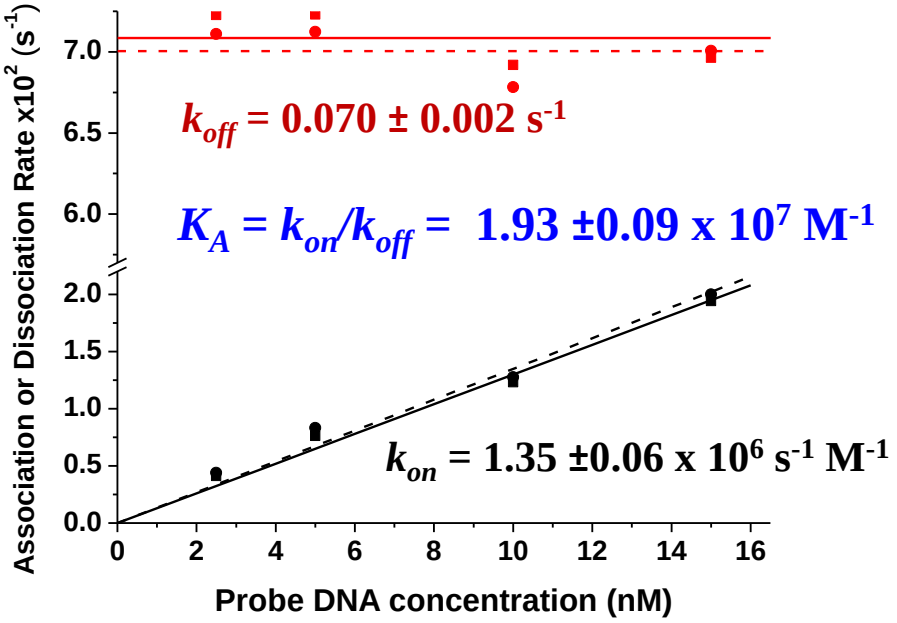


Histogram fit to Poisson-Erlang distribution

$$p(k,\lambda) = \frac{\lambda^k \exp(-\lambda)}{k!}$$

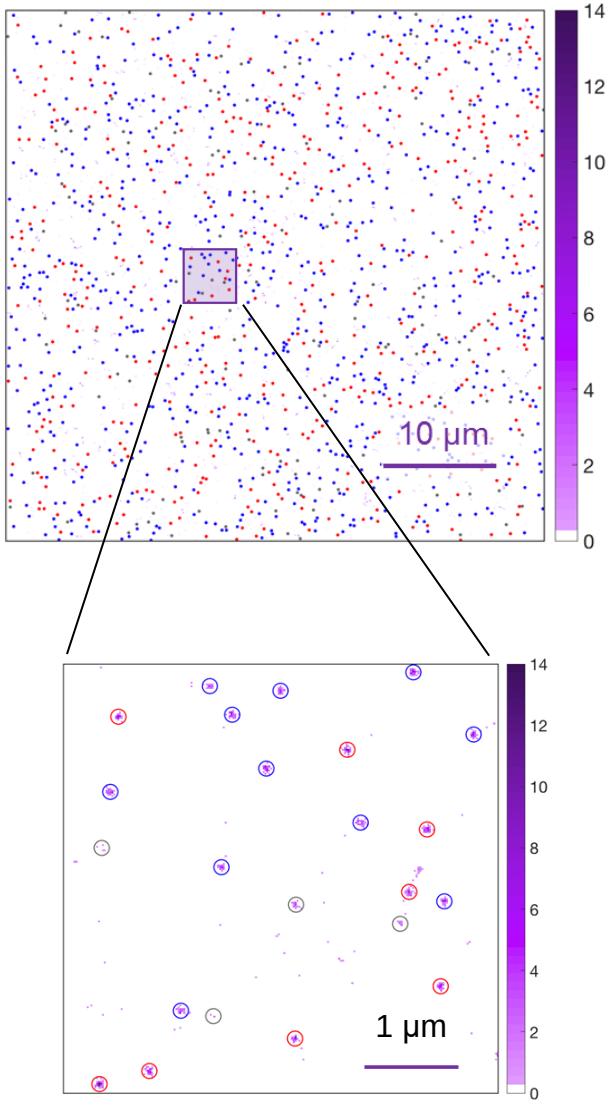
$$f(x;\lambda,\tau) = \sum_{k=1}^{k=\infty} p(k,\lambda) \frac{\left(\frac{k}{\tau}\right)^k x^{k-1} \exp\left(-\frac{xk}{\tau}\right)}{(k-1)!}$$

- Determine K_A directly from *kinetics*
- Precision in individual molecule kinetics largely governed by sampling statistics.
- Can *identify* molecules by their dissociation rates.



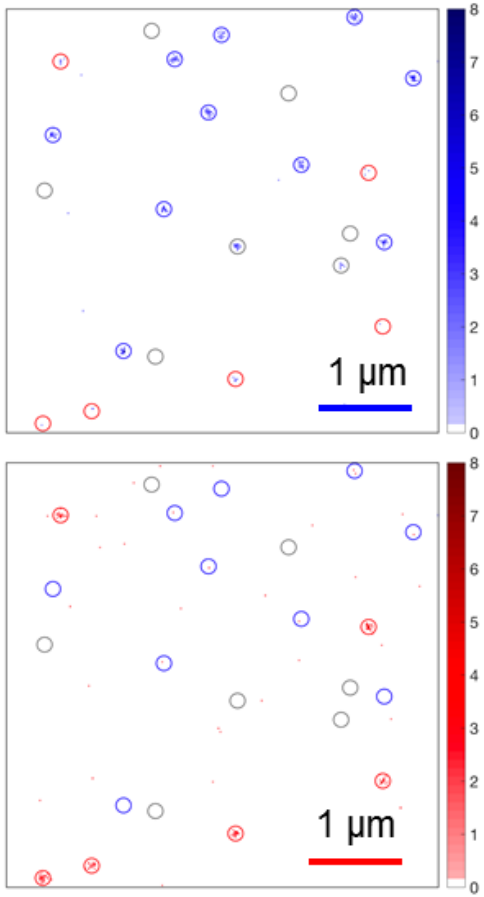
Identifying individual DNA molecules by dissociation kinetics

Mixed 5-nM T- and G-targets

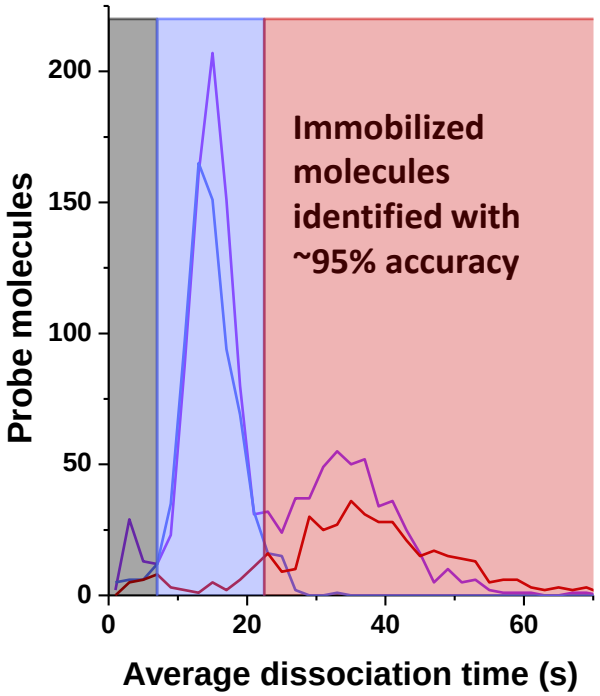


T-Probe	$(A)_{20}$-TTC GGT ATA TCC CAT
T-Target	A TAT AGG GTA-PEG_6-Cy3
G-Probe	$(A)_{20}$-TTC GGT ATA GCC CAT
G-Target	TAT CGG GTA-PEG_6-Cy3

10-nM T- and G-target standards

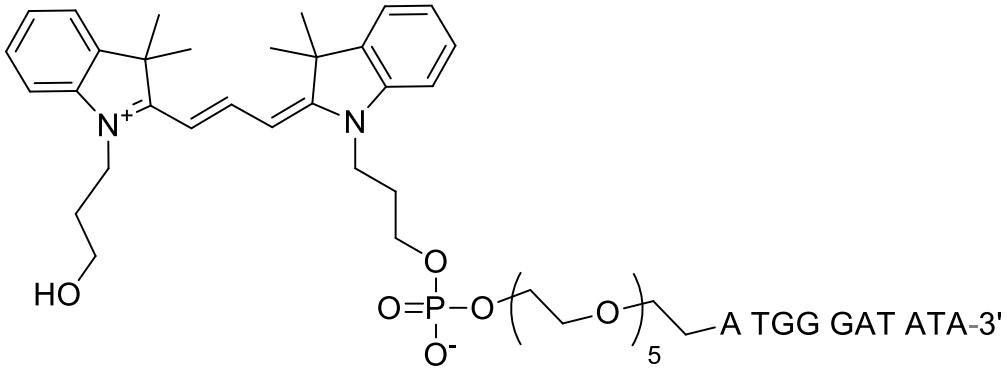


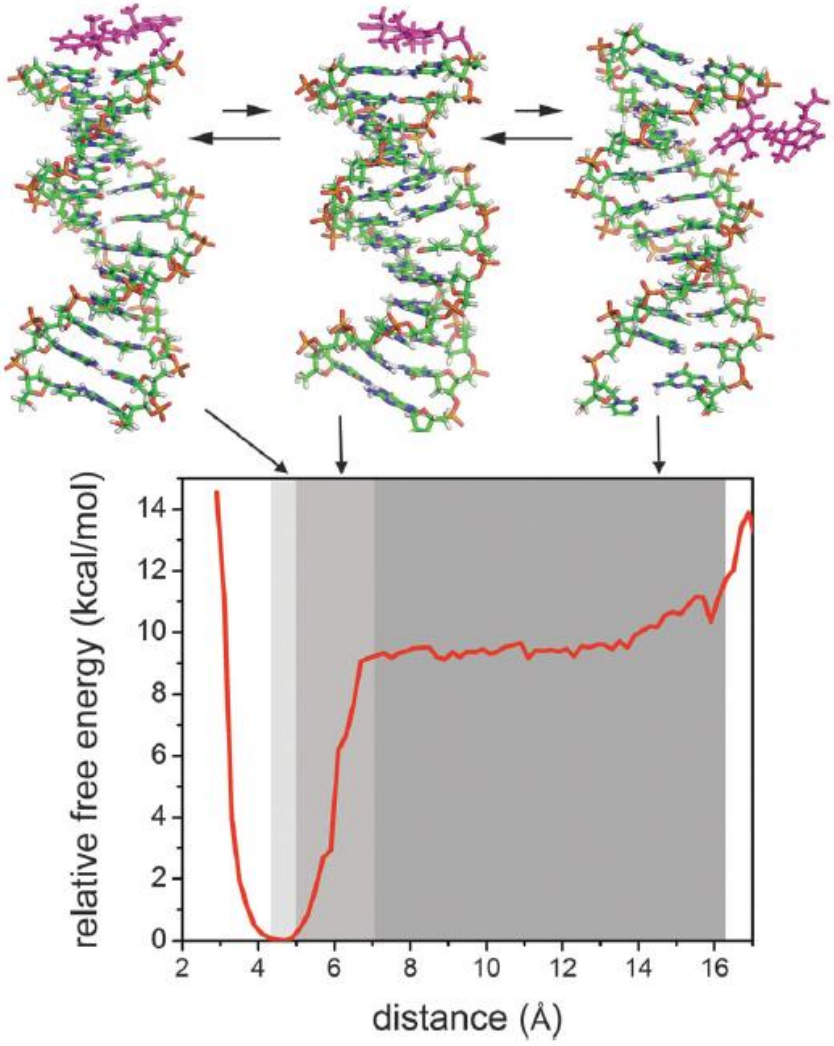
Distribution of dissociation times for immobilized probe molecules



Investigating effects of dye labels on DNA hybridization

- **Fluorescent labels interact with single- and double-stranded DNA**
- **Label impacts duplex stability by pi-stacking interactions: prevents fraying, strongest for A-T base pairs***



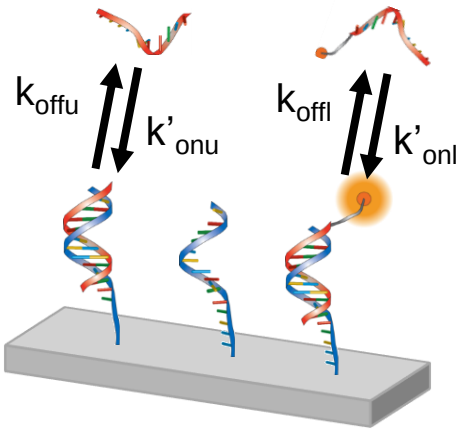


How can we measure the kinetics of *unlabeled* DNA hybridization for comparison?

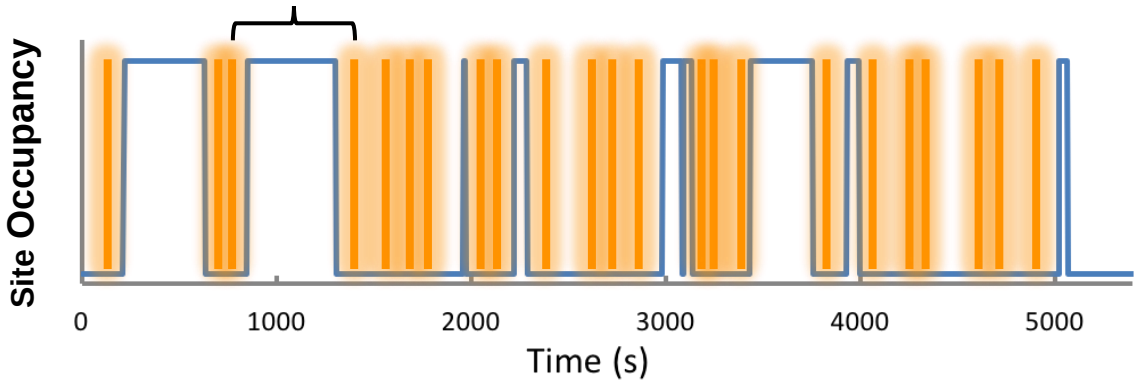
* Spiriti, J. ; Binder, J. K. ; Levitus, M. ; van der Vaart., A. *Biophys. J.* **2011**, *100*, 1049.

Observing single-probe sites: kinetics of competitive hybridization

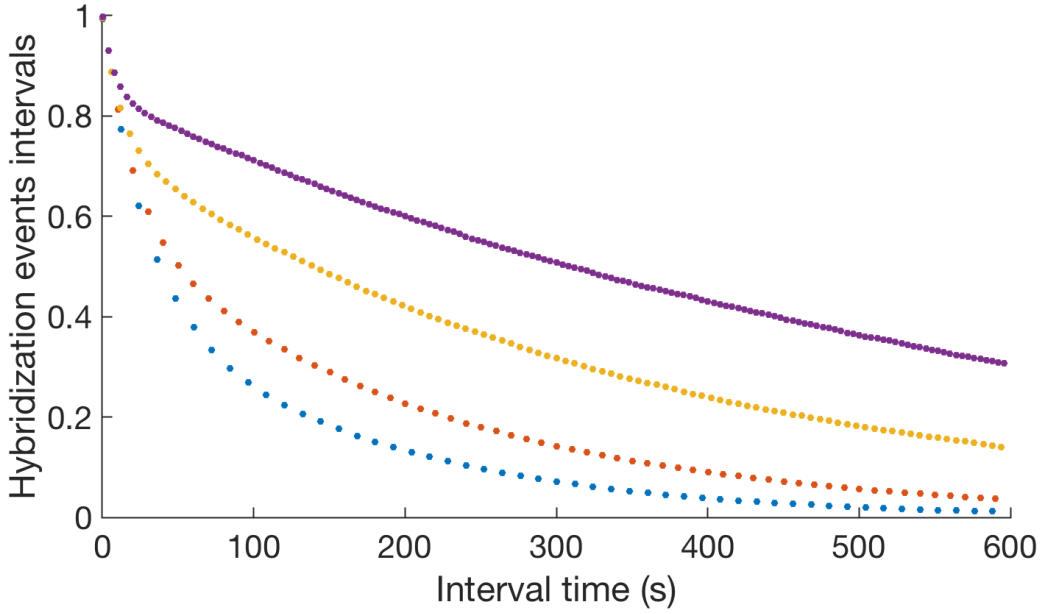
Competitive hybridization of labeled and unlabeled DNA



Association time of labeled DNA affected by unlabeled DNA kinetics



Monte-Carlo simulations of hybridization intervals



Simulated rates (s^{-1}):

$k'_{onl} = 0.022$

$k_{offu} = 0.01$

$k'_{onu} = 0.1$

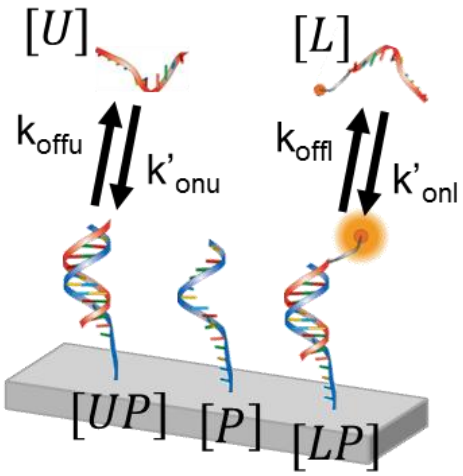
$k'_{onu} = 0.05$

$k'_{onu} = 0.02$

$k'_{onu} = 0.01$

Rate model for competing hybridization

Solve system of differential equations for competing hybridization



$$\frac{d[P]}{dt} = -k_{onu}[U][P] + k_{offu}[UP] - k_{onl}[L][P]$$

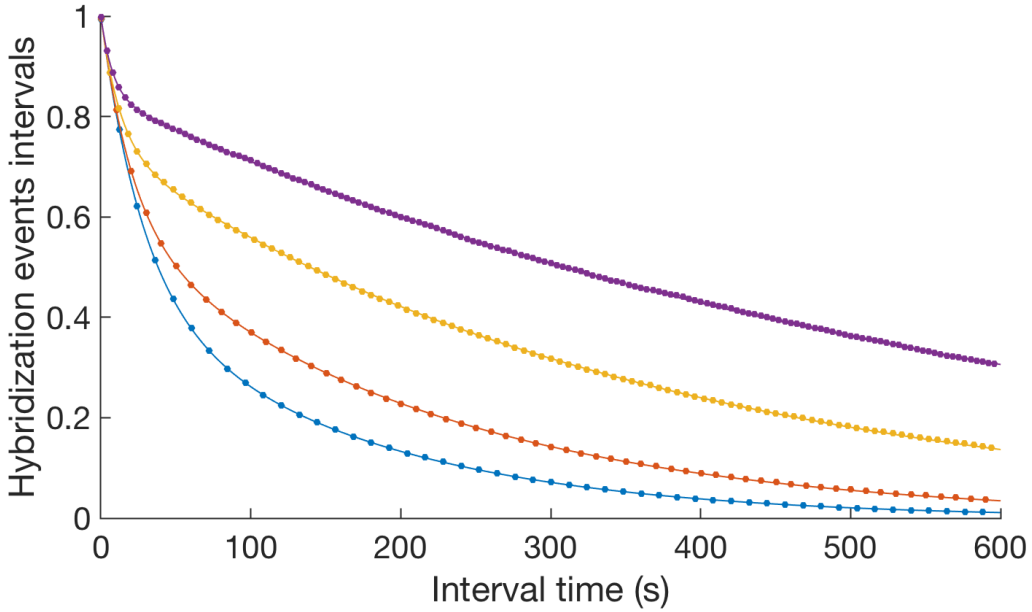
$$\frac{d[UP]}{dt} = k_{onu}[U][P] - k_{offu}[UP]$$

$$\boldsymbol{P(t) + UP(t) = P_{tot}\left[A_1 e^{-k_1 t} + A_2 e^{-k_2 t}\right]} \quad \text{where}$$

$$k_1 = \frac{k_{offu} + k_{onl} + k_{onu} + k_q}{2} \qquad k_2 = \frac{k_{offu} + k_{onl} + k_{onu} - k_q}{2}$$

$$k_q = \sqrt{k_{offu}^2 + k_{onl}^2 + k_{onu}^2 - 2k_{offu}k_{onl} + 2k_{offu}k_{onu} + 2k_{onl}k_{onu}}$$

Monte-Carlo simulations of hybridization intervals – fit to the model



Simulated rates (s^{-1}):	Rates from fit (s^{-1}) (±2%)
k'_{onl} = 0.022	k'_{onl} = 0.022
k_{offu} = 0.01	k_{offu} = 0.010
k'_{onu} = 0.1	k'_{onu} = 0.098
k'_{onu} = 0.05	k'_{onu} = 0.049
k'_{onu} = 0.02	k'_{onu} = 0.020
k'_{onu} = 0.01	k'_{onu} = 0.0099

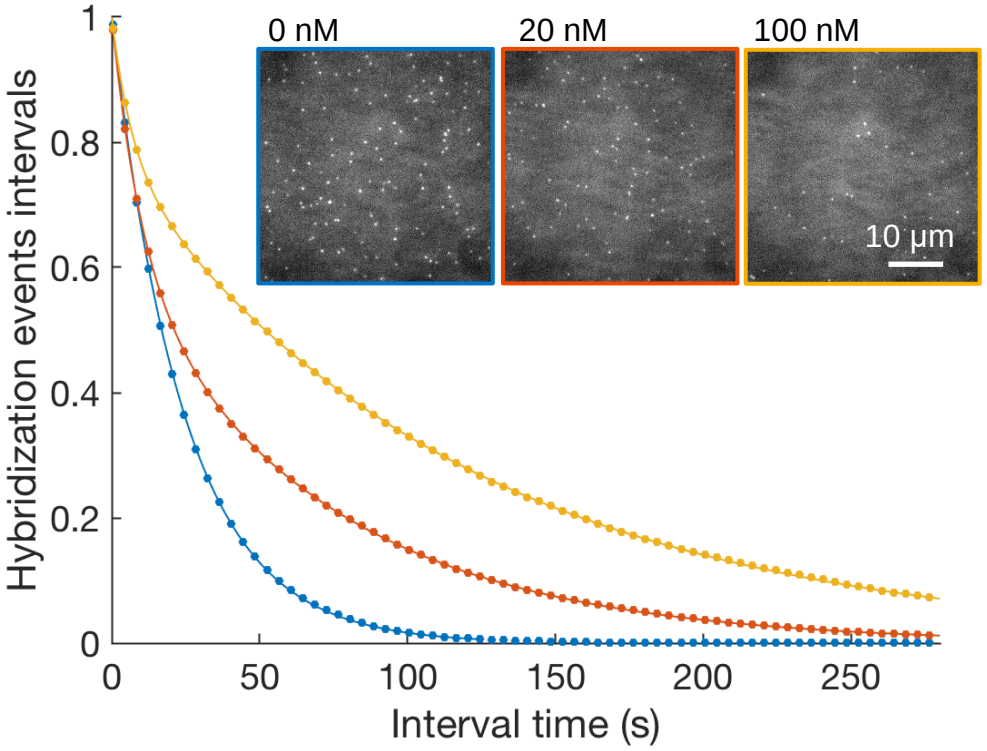
Experimental hybridization intervals

Experimental Conditions:

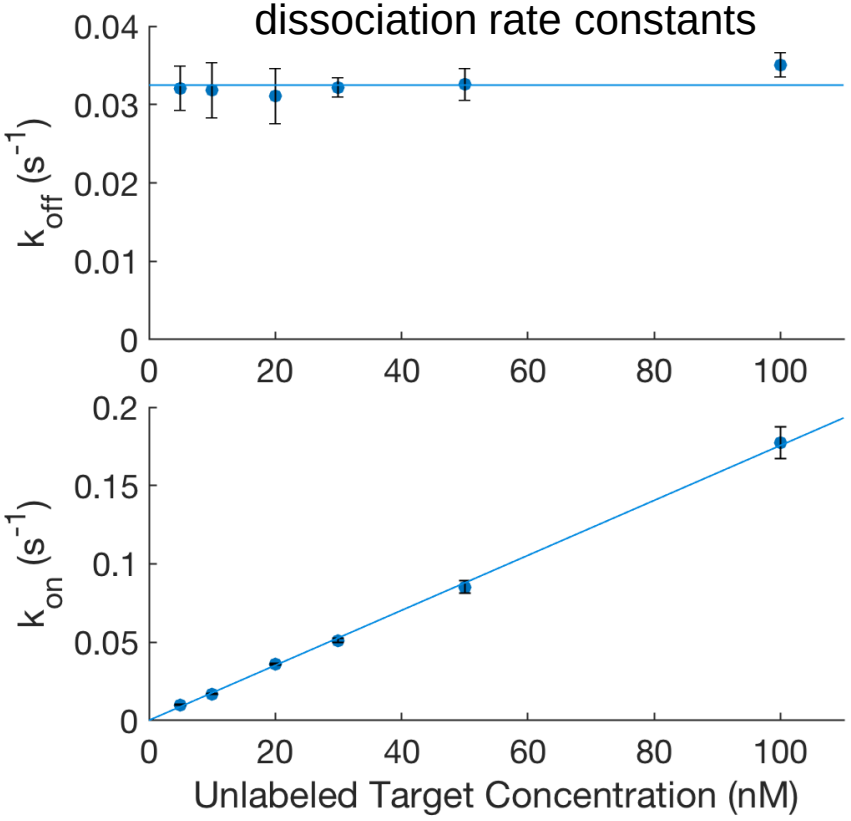
- Labeled target: 30 nM
- Unlabeled Target: (0-100nM)

Probe	5’-TTC GGT ATA TCC CAT
Labeled target	3’- TAT AGG GTA-Cy3
Unlabeled target	3’- CA TAT AGG GTA

Labeled target hybridization interval distribution



Unlabeled Target association and dissociation rate constants

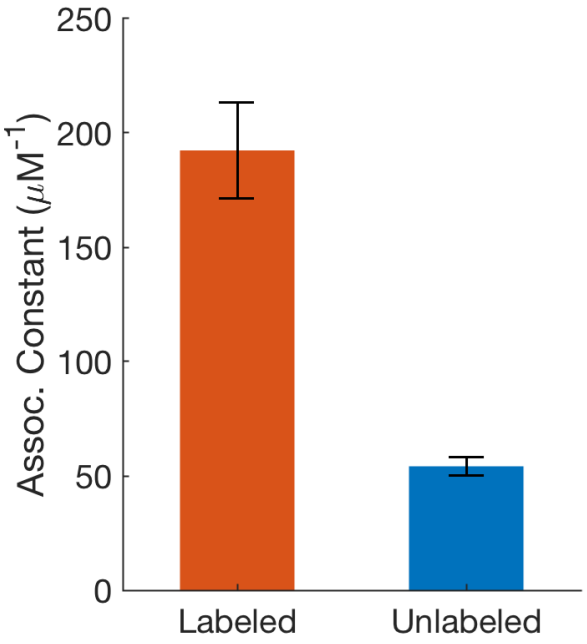


Impact of labeling on DNA hybridization kinetics

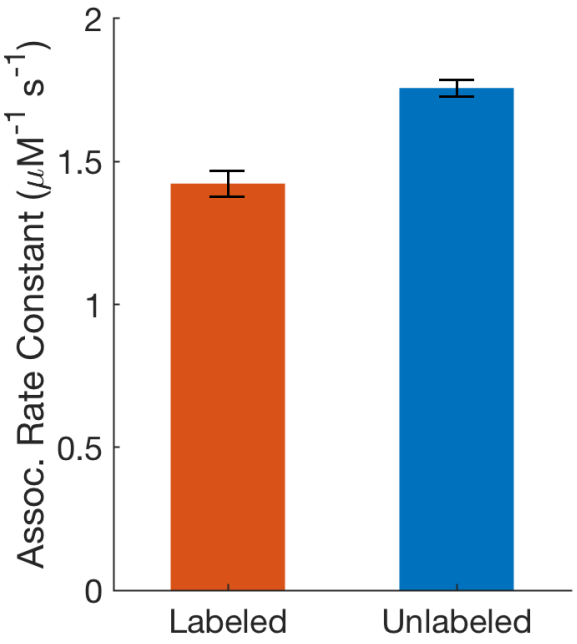
Fluorescent label adds significant stability to duplex

Probe	5’-TTC GGT ATA TCC CAT
Labeled target	3’- CA TAT AGG GTA-Cy3
Unlabeled target	3’- CA TAT AGG GTA

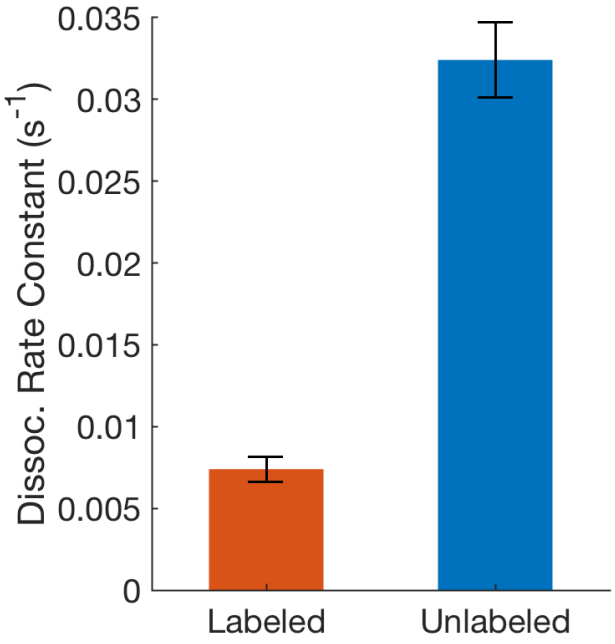
Association constant, K_a



Association rate constant, k_{on}



Dissociation rate constant, k_{off}



Labeling impacts both DNA duplex stability and kinetics

Summary comments

Measurements of interfacial binding equilibria and kinetics at the single-molecule level.

Quantification of probe DNA surface densities with single-molecule standards.

Measurement of DNA association constants, kinetics, competitive assays.

New directions: detecting single-nucleotide polymorphisms, assembly kinetics of DNA split-aptamers.

Acknowledgments

Eric Peterson Franki Morris Mike Manhart

National $cience Foundation