Published in final edited form as:

Angew Chem Int Ed Engl. 2017 February 20; 56(9): 2399–2402. doi:10.1002/anie.201612050.

Observing Single-Molecule Dynamics at Millimolar Concentrations

Marcel P. Goldschen-Ohm^{†,a}, David S. White^{†,a,b}, Vadim A. Klenchin^a, Baron Chanda^{a,c}, and Randall H. Goldsmith^b

^aDepartment of Neuroscience, University of Wisconsin-Madison, 1111 Highland Ave., Madison, WI 53705 (USA)

^bDepartment of Chemistry, University of Wisconsin-Madison, 1101 University Ave., Madison, WI 53706 (USA)

^cDepartment of Biomolecular Chemistry, University of Wisconsin-Madison, 420 Henry Mall, Madison, WI 53706 (USA)

Abstract

Single-molecule fluorescence microscopy is a powerful tool for revealing chemical dynamics and molecular association mechanisms, but was limited to low concentrations of fluorescent species only suitable for studying high affinity reactions. Here, we combine nanophotonic zero-mode waveguides (ZMW) with fluorescence resonance energy transfer (FRET) to resolve single-molecule association dynamics at up to millimolar concentrations of fluorescent species. This approach extends resolution of molecular dynamics to >100-fold higher concentrations, enabling observations at concentrations relevant to biological and chemical processes, and thus making single-molecule techniques applicable to a tremendous range of previously inaccessible molecular targets. We deploy this approach to show binding of cGMP to pacemaking ion channels is weakened by a slower internal conformational change.

Graphical abstract

Single-molecule resolution of association dynamics at millimolar concentrations of fluorescent ligands (>100-fold higher concentrations than previously accessible) with Zero-mode waveguides and FRET.

Correspondence to: Baron Chanda; Randall H. Goldsmith.

[†]These authors contributed equally.

Supporting information for this article is given via a link at the end of the document.

Author Contributions

V.A.K. performed all cloning and mutagenesis, protein purification and protein labeling. M.P.G. and D.S.W. performed all single-molecule experiments and analysis. M.P.G. developed all custom single-molecule image analysis software. M.P.G., R.H.G. and B.C. conceived and supervised the project. M.P.G., D.S.W., B.C. and R.H.G. wrote the manuscript.

Keywords

Cyclic nucleotide; FRET; Kinetics; Single-molecule studies; Zero-mode waveguide

Single-molecule fluorescence microscopy reveals details of molecular composition and dynamics otherwise hidden due to averaging in ensemble measurements.^[1] However, a frequent experimental compromise is the requirement of nM or lower concentrations of fluorescent species. This limitation originates from the diffraction-limit of focused light, as the smaller the probe volume can be made, the fewer molecules will contribute to background.^[1b] For example, confocal detection schemes with diffraction-limited excitation enable observation volumes as small as 0.1–0.2 femtoliters ($1 \text{ fL} = 1 \times 10^{-15} \text{ L}$).^[2] Thus, in both Confocal and Total Internal Reflection (TIRF) modalities, there is an inherent concentration limit of $<10 \text{ nM}$ to detect binding of a single fluorescently-labeled substrate.^[3] This “concentration barrier” is severely debilitating, as many biological and chemical mechanisms require highly concentrated conditions in the μM to mM range to proceed. Metabolites (including ATP), neurotransmitters, and amino acids are frequently present at cellular concentrations of $100 \mu\text{M}$ and above,^[4] preventing application of single-molecule microscopy to examine binding in kinases, receptors, translation machinery, and the vast majority of enzymes^[5] (Fig. 1a). Access to high concentrations becomes even more significant for new single-molecule investigations of synthetic catalysts, most of which operate at substrate or ligand concentrations of mM and above.^[6]

Increases to the highest attainable fluorophore concentration can be achieved through reduction in observation volume below the diffraction limit. Stimulated Emission Depletion (STED) in the context of fluorescence correlation spectroscopy (FCS) permits detection volumes near 0.02 fL .^[7] Photoactivation^[8] of or photobleaching^[9] down to sparse subsets of fluorophores transiently creates low concentrations of active fluorescent species from a larger reservoir of fluorophores enabling concentrations up to $10 \mu\text{M}$.^[10] Use of nanophotonic antennas have enabled access to up to $25 \mu\text{M}$ concentrations of fluorescent species^[11] with the added benefit of plasmonically enhanced fluorescence^[12]. Imaging in the vicinity of the interface between a convex lens and a flat surface enables access to concentrations up to $2 \mu\text{M}$.^[13] Indeed, a variety of chemical and photonic tools have enabled access to concentrations up to the low μM range^[14].

In particular, Zero-Mode Waveguides (ZMW), nanophotonic arrays of sub-wavelength holes in a metallic film (Fig. 1c), provide sub-diffraction-limited nearfield observation volumes as small as 20 zeptoliters ($1 \text{ zL} = 1 \times 10^{-21} \text{ L}$), far smaller than that achievable with TIRF or STED, such that single fluorophores can be resolved at up to low μM concentrations.^[2, 15] ZMWs have been successfully used to observe high nM to low μM molecular recognition processes including translation events at individual ribosomes,^[16] dynamics of membrane-bound proteins^[17], and single-molecule electrochemistry,^[18] and have enabled high-throughput single-molecule genomic sequencing.^[19] They have also been combined with plasmonic nano-antennas to gain the advantages of fluorescence enhancement^[20]. Regardless, association processes that require upwards of tens to hundreds of μM concentrations remain out of reach, thus requiring new single-molecule methods. Here, we

show that a combination of ZMWs and single-molecule FRET (smFRET) enables resolution of single-molecule molecular recognition events at mM concentrations. This approach merges ZMW's sub-diffraction-limited observation volume with a detection volume defined by the Förster radius of the FRET pair on the order of 1 zL (Fig. 1b, c).

Although smFRET alone enables observation of single-molecule binding dynamics at up to 10 μM ,^[21] access to higher concentrations was limited by non-specific adsorption and background from freely diffusing fluorophores. While both of these interferences exist in our dual ZMW-FRET method, their influence has been sharply reduced by the ZMW's volume restriction. Critically, our approach extends resolution of single fluorophore association by over 100-fold from low μM to low mM concentrations, allowing the elucidation of previously inaccessible biological and chemical mechanisms at the level of single molecules.

As validation, we report time-resolved single-molecule binding events for fluorescently-labeled cyclic guanosine monophosphate (fcGMP)^[22] to monomeric cyclic nucleotide-binding domains (CNBDs) from human hyperpolarization and cyclic nucleotide-activated (HCN) channels. HCN channels are critical for regulation of heart and brain rhythms, but the mechanism by which cyclic nucleotide binding modifies channel gating remains unclear. Single-molecule binding dynamics report on electrically silent and transient conformations energetically coupled to binding, and inform on the forces by which they interconvert, thus providing a novel window into this process. Here, we used ZMW-FRET to directly observe single binding events of fcGMP.

CNBDs were specifically labelled with a FRET acceptor and immobilized within arrays of over one hundred thousand ZMWs (Fig. 1c, and SI). Two EMCCD cameras were used to simultaneously record both donor (fcGMP) and acceptor fluorescence from $\sim 1,000$ ZMWs at once, making this approach feasible for high throughput studies. Excitation alternated between donor and acceptor pump wavelengths at the frame rate of 10 Hz, which allowed observation on interleaved frames of both smFRET due to donor binding (Fig. 2a, middle) and acceptor stability. Importantly, this method allows quantification of the number of fluorescently-labelled proteins in a ZMW by counting the number of acceptor bleach steps, thus enabling the selection of ZMWs featuring single proteins only (Fig. 2a, bottom). Notably, though individual bound fcGMP molecules could not be resolved at a concentration of 1 mM in the donor channel (Fig. 2a, top), the smFRET signal from single binding events was clearly visible in the acceptor channel prior to bleaching (Fig. 2a, middle). In comparison, direct observation of fcGMP binding without FRET was possible in ZMWs only at low μM concentrations (Fig. 2b).

Specific binding at single molecules as reported by smFRET was recorded at fcGMP concentrations from 1 μM to 1 mM (Fig. 3a). The concentration-dependence of the equilibrium bound probability across all molecules indicates an apparent affinity of $\sim 10 \mu\text{M}$ (Fig. 3b), similar to previous bulk-averaged measurements.^[23] Notably, binding curve saturation required hundreds of μM of fcGMP.

Histograms of bound and unbound dwell times were constructed from idealized pooled data (Fig. 4a, SI). As expected for a binding reaction, unbound lifetimes decreased with increasing fcGMP concentration, whereas bound lifetimes were relatively concentration independent. Based on previous observations that the CNBD isomerizes between two conformations,^[24] we compared the likelihood of several kinetic models (Fig. S2e). The preferred model had two unbound and two bound states, such that isomerization of the CNBD can occur both with and without bound ligand (Fig. 4b). This model is consistent with X-ray crystal structures in which the C-helix caps the bound ligand^[24–25], and electron paramagnetic resonance studies that suggest similar capping of the binding site also occurs in the unliganded CNBD, thereby temporarily blocking access by fcGMP.^[23] Furthermore, this scheme is similar to that observed for the binding dynamics of the higher affinity ligand fcAMP.^[24] In comparison to fcAMP at monomeric CNBDs, our high-concentration single-molecule studies reveal that the lower apparent affinity of fcGMP as compared to fcAMP is due not only to slower binding, but also to a reduction in the probability that bound ligand will induce a stabilizing isomerization of the CNBD that prolongs the total lifetime of the bound state.

Now that several methods allow access to elevated concentrations for single-molecule experiments, thus breaking the “concentration barrier” to varying degrees, it is useful to compare and contrast advantages of these methods. One issue relevant to investigation of biological structures is access to the biomolecule. Our ZMW-FRET combination, like other ZMW geometries, entails the biomolecule being in a microenvironment with a high surface-to-volume ratio, though the 100–150 nm diameter of the aperture is large compared to the CNBD and the sidewalls and bottom of the ZMW are passivated^[26]. This geometry is less restrictive than antenna based approaches that involve a nano-aperture^[11, 16] but more restrictive than photoactivation^[10] and FRET^[21] approaches that do not have the same constraints and may be more suitable for in vivo measurements, but. Our approach does not require specialized fluorophores, unlike photoactivation-based approaches^[10]. However, the temporal observation window of our approach as well as FRET-based approaches^[21] is limited by the photobleaching of the acceptor, whereas in ZMWs without FRET^[2, 15] or other approaches where all fluorophores are continually replenished^[10–11, 13, 16], the observation window will be longer, though higher stability fluorophores^[27] can likely extend the observation window in ZMW-FRET. Finally, only ZMW-FRET is capable of reaching the biologically significant range of 100 μ M–1mM.

In conclusion, we demonstrate single-molecule resolution of binding events at up to mM concentration, more than two orders of magnitude higher concentration than was feasible with other methods. Our combined ZMW-FRET approach enables observation of molecular dynamics at relevant concentrations for a vast majority of biological and chemical association reactions that were previously inaccessible to single-molecule techniques.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This research was supported by funding from the National Institutes of Health to B.C. (GM084140, NS081293) and D.S.W. (T32 GM007507) and National Science Foundation to R.H.G. (CHE-1254936). B.C. was also supported by Romnes Faculty fellowship (WARF). We thank Dr. Mike Sanguinetti for the wild-type HCN2 plasmid.

References

1. a) Ha T. *Nature Meth.* 2014; 11:1015–1018. b) Moerner WE, Fromm DP. *Rev Sci Instrum.* 2003; 74:3597–3619.
2. Levene MJ, Korlach J, Turner SW, Foquet M, Craighead HG, Webb WW. *Science.* 2003; 299:682–686. [PubMed: 12560545]
3. van Oijen AM. *Curr Opin Biotechnol.* 2011; 22:75–80. [PubMed: 21036593]
4. Bennett BD, Kimball EH, Gao M, Osterhout R, Van Dien SJ, Rabinowitz JD. *Nature Chem Bio.* 2009; 5:593–599. [PubMed: 19561621]
5. Scheer M, Grote A, Chang A, Schomburg I, Munaretto C, Rother M, Sohngen C, Stelzer M, Thiele J, Schomburg D. *Nucleic Acids Res.* 2011; 39:D670–676. [PubMed: 21062828]
6. a) Cordes T, Blum SA. *Nature Chem.* 2013; 5:993–999. [PubMed: 24256861] b) Ng JD, Upadhyay SP, Marquard AN, Lupo KM, Hinton DA, Padilla NA, Bates DM, Goldsmith RH. *J Am Chem Soc.* 2016; 138:3876–3883. [PubMed: 26944030] c) Roeffaers MB, De Cremer G, Uji-i H, Muls B, Sels BF, Jacobs PA, De Schryver FC, De Vos DE, Hofkens J. *Proc Natl Acad Sci U S A.* 2007; 104:12603–12609. [PubMed: 17664433]
7. Kastrop L, Blom H, Eggeling C, Hell SW. *Phys Rev Lett.* 2005; 94:178104. [PubMed: 15904340]
8. Manley S, Gillette JM, Patterson GH, Shroff H, Hess HF, Betzig E, Lippincott-Schwartz J. *Nature Meth.* 2008; 5:155–157.
9. Moertelmaier M, Brameshuber M, Linimeier M, Schütz GJ, Stockinger H. *Appl Phys Lett.* 2005; 87:263903.
10. Loveland AB, Habuchi S, Walter JC, van Oijen AM. *Nature Meth.* 2012; 9:987–992.
11. Puchkova A, Vietz C, Pibiri E, Wunsch B, Sanz Paz M, Acuna GP, Tinnefeld P. *Nano Lett.* 2015; 15:8354–8359. [PubMed: 26523768]
12. Kinkhabwala A, Yu Z, Fan S, Avlasevich Y, Mullen K, Moerner WE. *Nature Photon.* 2009; 3:654–657.
13. Leslie SR, Fields AP, Cohen AE. *Anal Chem.* 2010; 82:6224–6229. [PubMed: 20557026]
14. Holzmeister P, Acuna GP, Grohmann D, Tinnefeld P. *Chem Soc Rev.* 2014; 43:1014–1028. [PubMed: 24019005]
15. Zhu P, Craighead HG. *Ann Rev Biophys.* 2012; 41:269–293. [PubMed: 22577821]
16. Uemura S, Aitken CE, Korlach J, Flusberg BA, Turner SW, Puglisi JD. *Nature.* 2010; 464:1012–1017. [PubMed: 20393556]
17. Christensen SM, Triplet MG, Rhodes C, Iwig JS, Tu HL, Stamou D, Groves JT. *Nano Lett.* 2016; 16:2890–2895. [PubMed: 27013033]
18. Zhao J, Zaino LP 3rd, Bohn PW. *Farad Disc.* 2013; 164:57–69.
19. a) Korlach J, Bjornson KP, Chaudhuri BP, Cicero RL, Flusberg BA, Gray JJ, Holden D, Saxena R, Wegener J, Turner SW. *Methods Enzymol.* 2010; 472:431–455. [PubMed: 20580975] b) Larkin J, Foquet M, Turner SW, Korlach J, Wanunu M. *Nano Lett.* 2014; 14:6023–6029. [PubMed: 25209321]
20. Punj D, Mivelle M, Moparthy SB, van Zanten TS, Rigneault H, van Hulst NF, Garcia-Parajo MF, Wenger J. *Nature Nanotech.* 2013; 8:512–516.
21. Sugawa M, Nishikawa S, Iwane AH, Biju V, Yanagida T. *Small.* 2010; 6:346–350. [PubMed: 20033931]
22. Biskup C, Kusch J, Schulz E, Nache V, Schwede F, Lehmann F, Hagen V, Benndorf K. *Nature.* 2007; 446:440–443. [PubMed: 17322905]
23. DeBerg HA, Brzovic PS, Flynn GE, Zagotta WN, Stoll S. *J Biol Chem.* 2016; 291:371–381. [PubMed: 26559974]

24. Goldschen-Ohm MP, Klenchin VA, White DS, Cowgill JB, Cui Q, Goldsmith RH, Chanda B. *eLife*. 2016; 5
25. Zagotta WN, Olivier NB, Black KD, Young EC, Olson R, Gouaux E. *Nature*. 2003; 425:200–205. [PubMed: 12968185]
26. Korlach J, Marks PJ, Cicero RL, Gray JJ, Murphy DL, Roitman DB, Pham TT, Otto GA, Foquet M, Turner SW. *Proc Natl Acad Sci U S A*. 2008; 105:1176–1181. [PubMed: 18216253]
27. Zheng Q, Juette MF, Jockusch S, Wasserman MR, Zhou Z, Altman RB, Blanchard SC. *Chem Soc Rev*. 2014; 43:1044–1056. [PubMed: 24177677]

Figure 1. ZMW-FRET imaging

a, Histogram of over 30,000 enzyme affinities from the BRENDA database^[5].

Concentration ranges accessible to single-molecule resolution are indicated for several methods. **b**, Illustration of FRET between bound donor (fcGMP) and an acceptor on the CNBD. **c**, Experimental setup for ZMW-FRET microscopy. Fluorescence in donor and acceptor channels from arrays of ZMWs were simultaneously imaged on two EMCCD cameras. Inset: cartoon of a single ZMW with an immobilized CNBD. The observation volume decays rapidly within ~25 nm of the surface. A red circle represents the Förster radius, further reducing the effective observation volume. Thus, freely diffusing donors unbound to the CNBD are not observed.

Figure 2. Single-molecule ligand binding at mM concentrations with ZMW-FRET

a, Fluorescence time series for fcGMP binding events at a single CNBD with 1 mM freely diffusing fcGMP. Simultaneous emission from donor (blue, fcGMP) and acceptor (red) upon interleaved donor (532 nm) and acceptor (640 nm) excitation (see SI). Acceptor emission is overlaid with idealized time series (black). **b**, Fluorescence time series for fcGMP binding to CNBDs without an acceptor label (donor only). Notably, background from freely diffusing fcGMP in ZMWs occludes resolution of single binding events at high μM concentrations and above. Fluorescence time series in both **a** and **b** are background subtracted, while the smFRET trace in **a** additionally underwent crosstalk subtraction and baseline correction by spline fitting (see SI).

Figure 3. Single-molecule association dynamics of fcGMP at HCN CNBDs

a, FRET time series for fcGMP binding to single CNBDs (red) overlaid with idealized traces (black) at various concentrations of fcGMP. Traces shown underwent corrections in baseline with spline fitting and were both background and crosstalk subtracted (see raw traces in Fig. S4). Horizontal dashed lines indicate fluorescence levels for bound, unbound and bleached conditions. Triangle denotes time of acceptor bleach. **b**, Bound probability from total time fraction spent bound for all molecules vs. fcGMP concentration (circles) fit with $B_{\max}/(1+K_d/[fcGMP])$, where $B_{\max} = 0.83$ is the maximal bound probability and $K_d = 10 \mu\text{M}$ is the apparent dissociation constant (solid line). Prediction from the model in Figure 4b normalized to $B_{\max}$ is shown as a dashed line.

Figure 4. A dynamic model of fcGMP association at HCN CNBDs

a, Histograms of unbound and bound single-c molecule dwell time distributions (gray) for events from all molecules combined overlaid with monoexponential (blue dashed) or biexponential (red) maximum likelihood fits. Concentration of fcGMP for each pair of histograms is indicated on the left-most ordinate. Summary of fit parameters shown in Fig. S2a. **b**, Kinetic model of fcGMP association dynamics.