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proteins, these protein-protein interactions cannot be amplified. Single-molecule detection could thus be unique, ideal route for characterizing these 'inherently heterogeneous and tiny' protein-protein interactions. We here report on an antibody-based, label-free method that characterizes the signaling dynamics of native proteins. We have used our technique to characterize two core oncoproteins, KRas and EGFR in different samples including cancer cell lines, xenograft tumors, cancer-patient biopsy samples. We have characterized the single-molecule signaling kinetics and the proportion of single proteins that are actively binding with the downstream proteins tagged with fluorescent proteins. We also compare our analysis results, which are at the protein-protein interaction level, with the genotyping results of samples, which is the current standard for personalized medicine. Our results demonstrate the possibility that the single-molecule techniques could be a real analysis tool for clinically important samples, beyond cutting-edge tool for the curiosity-based science.

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Solubilisation of Lipid Membranes by Detergents: Probing the Three-State Model at the Single Vesicle Level

Steven D. Quinn, Lisa A. Deas, Paul A. Dalgarno, Gordon J. Hedley, Ifor D.W. Samuel, Carlos Penedo.
University of St Andrews, St Andrews, United Kingdom.

The solubilisation of lipid membranes by detergents is a common technique in the purification and isolation of proteins and in the study of membrane proteins. Biophysical studies of these solubilisation processes using ensemble-averaging techniques have provided an integrated picture of the solubilisation mechanism that involves three states: the detergent is taken up in stage I without solubilisation (vesicle regime); stage II refers to the coexistence of detergent-saturated membranes and mixed detergent-lipid micelles and finally stage III corresponds to complete membrane solubilisation leading to the formation of mixed micelles (micellar regime). Although the three-state hypothesis is a didactic and simple thermodynamic model, it is known that detergent lipid-interactions induce a much more complex and diverse dynamic transition between both regimes. These dynamic changes include transmembrane lipid motion (flip-flop), swelling and breakdown of the membrane permeability barrier and fusion between vesicles. To date, whether these processes are independent of each other and take place sequentially, or if they are somehow interconnected is an open question. Here, we have used single-molecule FRET to monitor the solubilisation dynamics of single POPC/POPS vesicles induced by the non-ionic detergent Triton-X 100. Using this approach we have been able to unambiguously separate within a single FRET trajectory the swelling, permeabilization and lysis steps of the solubilisation process and their kinetic details above and below the critical micellar concentration. The present strategy should help in the design of more efficient applications of vesicle solubilisation.

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Long-Term Single-Molecule TIRF Observation of Biomolecules without Immobilization

Swati Tyagi, Virginia Vandelinder, Niccolo' Banterle, Gustavo Fuertes Vives, Morgane Agez, Sigrid Milles, Edward A. Lemke.
EMBL, Heidelberg, Germany.

Observation of individual molecules for long stretches of time aids in extracting dynamics and reveal hidden heterogeneity in biological systems. Commonly this is achieved by utilizing the high optical sectioning power of total internal reflection (TIR) microscopy, which involves complex immobilization procedures that cannot be generally applied and often perturb the native state of the biological sample. Here we present an easily adaptable and low-cost nanofluidic technology that enables us to perform long-term observation of freely diffusing single molecules. The microfluidic device is fabricated with soft lithography and molding techniques out of an elastomeric material, which allows highly reproducible production of several devices per day. Biomolecules flow through channels that are less than 100 nm deep, which keeps them within the TIR field. The nanochannels are actively generated by collapsing 1 µm deep flow channels using pressurized nitrogen gas in a control channel, which additionally increases photo stability of fluorophores by removing oxygen. We demonstrate that several second long time trajectories of thousands of molecules can be recorded with millisecond time resolution, illustrating the potential for long-term automated and high throughput experiments. We also show that the compatibility of the device with large biological complexes by showing multi-channel FRET imaging of freely diffusing single nucleosomes.

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Accurate Intramolecular Distances by Single Molecule Confocal Spectroscopy: A Monte Carlo Markov Chain Analysis of Fluorescence Data from Freely Diffusing Biomolecules

Rebecca R. Murphy[1], George Danezis[2], Sophie E. Jackson[1], David Klenerman[1].
[1]University of Cambridge, Cambridge, United Kingdom, [2]Microsoft Research Cambridge, Cambridge, United Kingdom.

Förster Resonance Energy Transfer (FRET) is a powerful technique for studying the conformational dynamics of biological systems at the level of single molecules. FRET experiments on single molecules (smFRET) in solution have the potential to recover accurate intramolecular distances. However, established methods for event selection and de-noising of smFRET data, are *ad hoc* and cause systematic biases in the results obtained.
We introduce a novel method for the analysis of smFRET data, suitable for the study of protein folding or molecular association. The technique is based on a simple stochastic model of the physical FRET process. Based on this model, we devise a Bayesian inference technique to infer directly all key features of a smFRET dataset, using Markov Chain Monte Carlo (MCMC) sampling methods. The proposed technique is computationally fast, requires only a few minutes of data collection, yields reliable confidence intervals, and requires no subjective choice of parameters for de-noising.
We evaluate the technique's performance using a combination of synthetic and experimental data, demonstrating that the method:
i. effectively identifies fluorescence events;
ii. distinguishes noise and fluorescence contributions to fluorescence events;
iii. infers simultaneously the rate parameters for the noise distribution, the FRET efficiencies, and relative sizes of one or more fluorescent populations.
We validate the technique on smFRET data from mixtures of well characterised dual-labelled DNA duplexes. Our analysis method is able to infer both the mean FRET efficiencies of multiple FRET populations and the relative sizes of these populations. Results will be presented demonstrating that our Bayesian inference technique obtains accurate intramolecular distance information from smFRET experiments, whereas established techniques fail.

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Optimal Estimation of Diffusion Coefficients from Noisy Single-Particle Trajectories

Christian L. Vestergaard[1], Paul Blainey[2], Henrik Flyvbjerg[1].
[1]Technical University of Denmark, Kgs. Lyngby, Denmark, [2]MIT Department of Biological Engineering and Broad Institute, Cambridge, MA, USA.

Super-resolution microscopy allows us to track single molecules in cells. There, diffusion is ubiquitous, as many cellular processes rely on diffusion for transport. A precise understanding of such processes requires a precise determination of diffusion constants. Less than that may miss process-specific details by lumping them into a single, simple diffusive process as we demonstrate here. We present a simple, optimal, unbiased estimator of diffusion coefficients of freely diffusing particles in one, two, or three dimensional homogeneous media. It takes time-lapse recorded single-particle trajectories as input, is vastly superior to estimates based on the mean squared displacement as function of time, and is superior to Maximum Likelihood estimation for short trajectories. We extend the method to diffusion on one-dimensional fluctuating substrates. As a pertinent practical illustration of the power of these tools, we use them to reveal multi-state kinetics in the diffusion of a protein on flow-stretched DNA, a fluctuating substrate.

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In Silico, In Vitro, and In Vivo Estimation of J-Factors for LAC Repressor-Mediated DNA Loop Formation

Sandip Kumar[1], David Priest[2], Yoav Biton[3], David Swigon[3], Keith Shearwin[2], Ian Dodd[2], David Dunlap[1].
[1]Emory University, Atlanta, GA, USA, [2]University of Adelaide, Adelaide, Australia, [3]University of Pittsburgh, Pittsburgh, PA, USA.

Transcription is often regulated by regulatory proteins with specific binding sites that can be many kilo-bases away from promoters. This is made possible by long range, DNA looping interactions. According to the ''loop domain model'', the separation of promoters and binding sites into separate topological domains can block promoter-regulator communication and this has been shown in bulk, *in vitro* experiments. However, the efficiency and regulation of long-range DNA looping are not well understood. To investigate looping, simulations, tethered particle motion (TPM) experiments, and measurements of reporter gene repression were used to estimate *J*-factors (J_{loop}) for lac