Observing conformations of single F₀F₁-ATP synthases in a fast anti-Brownian electrokinetic trap
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ABSTRACT

To monitor conformational changes of individual membrane transporters in liposomes in real time, we attach two fluorophores to selected domains of a protein. Sequential distance changes between the dyes are recorded and analyzed by Förster resonance energy transfer (FRET). Using freely diffusing membrane proteins reconstituted in liposomes, observation times are limited by Brownian motion through the confocal detection volume. A. E. Cohen and W. E. Moerner have invented and built microfluidic devices to actively counteract Brownian motion of single nanoparticles in electrokinetic traps (ABELtrap). Here we present a version of an ABELtrap with a laser focus pattern generated by electro-optical beam deflectors and controlled by a programmable FPGA. This ABELtrap could hold single fluorescent nanobeads for more than 100 seconds, increasing the observation times of a single particle by more than a factor of 1000. Conformational changes of single FRET-labeled membrane enzymes F₀F₁-ATP synthase can be detected in the ABELtrap.

Keywords: ABELtrap, Brownian motion, F₀F₁-ATP synthase, single-molecule FRET.

1. INTRODUCTION

The focus of our research for nearly 20 years has been the unravelling of conformational dynamics of the membrane-embedded enzyme F₀F₁-ATP synthase¹-³⁴ which is driven by two distinct rotary motors. This ubiquitous nanomachine in the thylakoid membrane of plant cells, in the inner mitochondrial membrane of eukaryotes and in the cytoplasmic membrane of bacteria catalyzes the chemical synthesis of adenosine triphosphate (ATP) from adenosine diphosphate (ADP) and phosphate Pᵢ. Therefore, it converts the chemical energy provided as a proton (or Na⁺ in some organisms) concentration difference plus an electric potential across the membranes into mechanical energy of subunit rotation, and the rotary double motor assembly forces and synchronizes the opening and closing of the catalytic ADP and Pᵢ binding sites where ATP is synthesized³⁵-⁵⁸.

We apply a single-molecule biophysics approach to study the bacterial F₀F₁-ATP synthase from Escherichia coli (and other membrane transporters⁶,¹⁷,³⁹-⁵²). The analysis of rotary conformational dynamics of the enzyme is achieved by single-molecule Förster resonance energy transfer (smFRET). We modified the protein by introducing cysteines or fusion of fluorescent proteins at different sites of the F₁ part or the membrane-embedded F₀ part, respectively. Thereby, we can specifically attach one fluorophore at the rotary subunits γ or ε in F₁, or ε in F₀. The second fluorophore for smFRET is attached to a static subunit, and rotary catalysis is observed as sequential stepwise distance changes between the two marker dyes. The FRET-labeled enzymes are reconstituted as single proteins in artificial liposomes with diameters in the range of 120 to 150 nm. Adding either Mg-ATP for ATP hydrolysis, or Mg-ADP and Pᵢ plus generating an electric potential across the membrane for ATP synthesis, respectively, results in FRET changes. Depending on the respective motor properties, i.e. the F₁ motor with the γ / ε rotor stepping in 3 steps (or, with sub-steps, 6,⁵³-⁵⁴ or 9,⁵⁵), or the rotating ring of c-subunits in F₀, in 10 steps, different numbers of FRET level occur and can be discriminated by FRET efficiencies and / or dwell times of the level.

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Confocal smFRET using freely diffusing proteoliposomes generates photon bursts which have a limited mean observation time for a proteoliposome to traverse the excitation focus and exhibit strong intensity fluctuations within a photon burst due to stochastic Brownian movement through a three-dimensional Gaussian excitation and detection profile. Both limitations, i.e. short time trajectories and partly low and fluctuating intensities, makes the use of likelihood estimators for FRET level or change point analysis necessary. We use Hidden Markov Models and, in part, also the a priori knowledge of the symmetry of the two motors to analyze the stepping behavior in a more reliable way than manual inspection and level assignment. As our attempts to immobilize the proteoliposomes with FRET-labeled F$_{o}$F$_{1}$-ATP synthase on a cover glass surface did not yet yield fully functional enzymes, we were looking for other options to investigate this nanomachine in solution, but on longer time scales and with constant fluorescence intensities for the sum of FRET donor and FRET acceptor dyes.

Exactly ten years ago, Adam E. Cohen and W. E. Moerner presented a novel microfluidic device that could actively compensate the Brownian motion of small particles like 20-nm polystyrene beads in water and, thereby, achieved prolonged observation times (more than 1000-fold). This device was called 'Anti-Brownian electrokinetic trap' or ABELtrap. It comprises a less than 1 µm shallow optically transparent microfluidic structure (made of PDMS, glass or quartz, respectively) to prevent diffusion in z-direction. Four access channels lead to this cross-like trapping region. In each of these access channels with a height of more than 20 µm, a Pt-electrode is positioned. Fluorescence of the molecule or nanoparticle is used to estimate its position within the trapping region in real time providing the fast feedback possibility. Depending on the actual x- and y-distances to a target position, voltages are supplied to the respective Pt-electrodes, and the fluorescent object is moved to the target position by both electrophoretic and electroosmotic forces. A. E. Cohen and W. E. Moerner quickly improved the trapping technology from EMCCD image- and software-based localization for the electro-kinetic feedback to the fastest FPGA-controlled hardware in only a few years. Nowadays, single fluorophores in solution can be trapped and their photophysics studied in unprecedented detail.

Directly after the remarkable ABELtrap oral presentation at SPIE Photonics West 2005, the author (M. B.) approached A. E. Cohen and W. E. Moerner to ask for a collaboration to study rotation of the FRET-labeled F$_{o}$F$_{1}$-ATP synthase at work in the ABELtrap at Stanford, and to learn how to build such an important tool for smFRET studies. Today, we report the status of our efforts to setup a fast ABELtrap in our group and to hold a single FRET-labeled F$_{o}$F$_{1}$-ATP synthase in an ABELtrap successfully. Therefore, this short progress report is gratefully dedicated to the ABELtrap pioneers and collaborators Adam and W. E..

2. EXPERIMENTAL PROCEDURES

2.1 Microscope setup and sample chamber preparation (PDMS microfluidics)

We built a FPGA-controlled ABELtrap as published by A. Fields and A. E. Cohen in 2011, with minor modifications. Laser excitation was provided by a continuous-wave laser at 491 nm (Cobolt Calypso, 50 mW) that was attenuated to 100 to 300 µW. The laser beam was steered by a pair of electro-optical beam deflectors, EOBDs (Model 310A, Conoptics) and directed to the back aperture of the microscope objective by a lens (f = 1000 mm). We used a TIRF 100x oil immersion objective with a numerical aperture of 1.49 (Olympus) mounted in an Olympus IX71 inverted microscope, and a dichroic beam splitter (z488xrd, AHF Tübingen) to reflect the laser and to block backscattered light from the ABELtrap. Two single photon-counting APDs recorded the fluorescence intensities in two spectral ranges from 500 to 570 nm (HQ535/70, AHF) and from 595 to 665 nm (HQ630/70, AHF), separated by an imaging beam splitter at 580 nm (BS580, AHF). Both APDs were mounted on a single 3D-adjustable mechanical stage (OWIS, Germany) that also contained the 150 µm pinhole.

Photons were recorded in parallel on two computers, i.e. by a FPGA card (PCIe-7852R, National Instruments) and by two synchronized TCSPC cards (SPC150 and SPC150N, respectively, Becker&Hickl, Germany), similar to our different ABELtrap setup published previously. The FPGA Labview program from A. Fields and A. E. Cohen was slightly modified to allow for trapping on the signals of either one or on both of the two detection channels. The controls for the 3D piezo sample scanner (P-527.3CD with digital controller E-725.3CD, Physik Instrumente, Germany) were adapted in the FPGA software as well. For calibration purposes, single fluorescent nanobeads were placed on a cover glass by spin coating, and were moved stepwise by the x-y-z piezo stage.

The microfluidic PDMS design we used was published previously, and was similar to the original design by A. E. Cohen and W. E. Moerner. Sylgard 184 elastomer kit (Dow Corning, Farnell, Germany) was used to fabricate the PDMS chambers for the ABELtrap. Short plasma treatment of both PDMS chip and cover glass resulted in irreversible bonding.
3. RESULTS

3.1 PDMS and quartz chips, illumination pattern, calibration of the ABELtrap

The ABELtrap requires microfluidics to prevent diffusion in z-dimension. The microfluidic chips consist of either a combination of structured PDMS bonded to the cover glass, or can be made as all-quartz cells. We previously established the PDMS/glass chips using a structured silicon wafer as the template for the PDMS polymerization. We have recently started to make all-quartz cells for significantly reduced luminescence background. In Fig. 1, the design of the two masks for both the shallow trapping region (1 µm thin) and the deeper access channels with ring structure for balancing hydrostatic pressure differences (20 µm deep) are shown.

For the ABELtrap experiments with the setup described in the following, we used our previous PDMS/glass chips. The chips have a similar design of the trapping region, but deeper access channels (~80 µm). PDMS/glass chips are cheap to produce and disposable. The drawback is a high luminescence background from both the cover glass in a spectral region above λ=600 nm, and fluorescent impurities in the PDMS.
Fluorescent nanoparticles or proteoliposomes with fluorescently labeled F$_{43}$-ATP synthases were excited by a continuous-wave solid state laser with 491 nm emission. To scan the laser beam over an area of 2 μm times 2 μm in the sample plane that was placed within the shallow region of the ABELtrap chip, we used two electro-optic beam deflectors (Model 310A, Conoptics) with an achromatic λ/2 wave plate in between (RAC 3.2.10, B. Halle Nachf., Germany). EOBDs were driven by two fast amplifiers (Model 7602M, Krohn-Hite). Using two plano-convex lenses (f = 80 mm) we imaged the first EOBD deflection plane onto second one, which was then projected onto the back-focal plane of the 100x microscope objective (N.A. 1.49, oil immersion, Olympus) by a f = 1000 mm lens (LA1779-A, Thorlabs).

The FPGA Labview software to run the ABELtrap is distributed freely by A. Fields and A. E. Cohen. We selected the circular pattern of 19 focal points and chose a diameter of the focus pattern of about 0.8 μm. Our expanded laser focus size was ~1.3 μm according to FCS measurements of diffusing dye molecules and to the EMCCD camera image of a 20-nm bead attached to the cover glass surface, respectively.

To optimize the optical alignment and to test the operation of our feedback system, we used immobilized 100-nm fluorescent beads which were scanned through the trapping region. The x- and y-feedback voltages were recorded for each position. Care was taken to ensure that the position to voltage-mapping was linear in the central part of the trapping region. We confirmed the linearity of feedback voltage vs. position that was sufficient in a range of roughly 0.9 x 0.9 μm (Fig. 2 A). The covered area of the laser pattern in the trapping region matched the used confocal pinhole with 150 μm diameter in the detection path.

3.2 Holding 20-nm fluorescent beads in aqueous solution in the ABELtrap

The performance of the ABELtrap depends on the precision of the EOBD-driven fast laser pattern (here with adjustable repetition rates for each pattern between 0 and 66 kHz), the accuracy of the estimated localization of the fluorescent particle or molecule, and the response time of the applied feedback voltages. We evaluated the ABELtrap with bonded PDMS/glass chips using diluted suspensions of fluorescent nanobeads with 20 nm in diameter, *i.e.* FluoSpheres 505/515 or Fluospheres Nile Red 535/585 (with given absorbance and emission maxima, Molecular Probes) in water in the presence of 0.1% polyvinylpyrrolidone (PVP, M₉ 40000, Sigma-Aldrich). Dilution and final concentration of the nanobeads were checked by FCS measurements in solution.

The mean fluorescence brightness of the FluoSpheres 505/515 excited with 491 nm was very high so that we could reduce the laser power and, thereby, achieved a very low background count rate of less than 2 counts per ms (< 2 kHz). Nanobeads showed distinct brightnesses as expected, but mean photon count rates of 200 to 300 per ms were regularly detected. In Fig. 2 B, ABELtrapping of a single 20-nm bead is shown resulting in a residence time in the trap of more than 40 seconds. The time trace exhibited fluctuating intensities due to minor imperfections of the alignment of the ABELtrap.

The beginning of the intensity trace of the trapped nanobead is expanded in Fig. 2 C. At a measurement time of at 6 s, the photon count rate rose in one step from background level to the mean brightness level of this particular bead of about 120 kHz. The simultaneously recorded electrode voltage time traces fluctuated only between ± 2 V once the bead was trapped, but oscillated stochastically between ±5 V at times where no bead was trapped. Because the voltage feedback is applied for each detected photon also in the absence of a trapped fluorescent molecule (*i.e.* from background of the PDMS/glass chip, or ‘dark counts’ from the APDs), a corresponding apparent ‘position’ is estimated for each photon detection event. Because these ‘positions’ are immediately changing throughout the trapping area, the applied feedback voltages will cover all allowed minimum and maximum values. Symmetrical voltage fluctuations around 0 V during the residence time of the bead were interpreted as strong indications for trapping of a bead in solution and not of a surface-sticking bead, because the position of a surface-sticking nanoparticle will be likely out-of-center of the trapping region, and, as a consequence, feedback voltages will exhibit a permanent deviation from fluctuations around 0 V for one or both x- and y-direction related electrodes.

The ABELtrap software also records the estimated deviation from the center of the ABELtrap as a time trace shown in Fig. 2 E for this nanobead. In the beginning of the time trace, *i.e.* in the absence of the bead, the apparent deviation from the center was fluctuating up to 250 nm. However, the estimated position of the trapped nanobead was deviating from the center only up to 50 nm, *i.e.* the trapped bead was confined to less than 100 nm. This confinement of a 20-nm bead was a significant improvement compared to the position fluctuations of trapped nanobeads as measured in our previous ABELtrap versions.
Figure 2: A. illumination pattern of the laser as detected by the fluorescence of a surface-attached 20-nm bead (Fluosphere 505/515). B. time trace of a trapped 20-nm bead in aqueous solution. The time binning was 10 ms. C-E, details of the time trajectories for this bead at the beginning of trapping, with 10 ms time binning. C. fluorescence intensity trace. D. electrode voltage time trace. E. estimated position deviation time trace plotted as distance from center, for the bead trapped at 6 s.

3.3 Holding proteoliposomes with labeled FₐFₐ-ATP synthases in buffer solution in the ABELtrap

After alignment of the ABELtrap and initial performance tests with 20-nm beads, we started trapping of proteoliposomes comprising one or more Alexa488-labeled FₐFₐ-ATP synthases. This preparation of reconstituted, labeled FₐFₐ-ATP synthases exhibited significantly high ATP synthesis rates measured in the biochemical Luciferin / Luciferase assay, i.e. the cysteine mutation at the C-terminus of the membrane-embedded subunit a did not interfere with the catalytic activity of the enzyme nor the subsequent covalent attachment of the Alexa488 dye. In this preparation we intended to embed more than a single labeled FₐFₐ-ATP synthase on average into the lipid bilayer of a liposome. Therefore, we expected multiple
fluorophores and, correspondingly, distinct mean photon count rates for each individual proteoliposome. As seen in the fluorescence intensity time trace in Fig. 3 A, trapping a single proteoliposome resulted in different intensity levels. Some trapped proteoliposomes showed stepwise photobleaching and strong intensity fluctuations (for example, see trace at time 152.5 s). The residence time of the proteoliposomes often approached 1 second, in contrast to a mean diffusion time of only 30 ms when the ABELtrap was turned off. In some proteoliposomes the fluorescence intensities were found nearly constant (for example, see trace at time 151.5 s).

**Figure 3:** A. time trace of ABELtrapped proteoliposomes with one and more Alexa488-labeled F$_0$F$_1$-ATP synthases. Trapping was achieved using Alexa488 fluorescence photons. Note that this intensity trace is shown without background subtraction. B, time trace of ABELtrapped proteoliposomes with single FRET-labeled F$_0$F$_1$-ATP synthases with Alexa488 as FRET donor (blue trace) and Atto594 as FRET acceptor (green trace). Trapping was achieved using Atto594 fluorescence photons. Note that this intensity trace is shown with background subtraction in both channels. FRET efficiencies were calculated per time bin as the proximity factor P (time trace in upper panel) without further corrections. Mean FRET efficiencies in selected photon bursts were assigned to low FRET (L), medium FRET (M) and high FRET (H) level.
Finally we explored the possibility of trapping a single FRET-labeled F$_6$F$_{17}$-ATP synthase in a liposome with a mean diameter of 120 nm. Shown in Fig. 3 B are preliminary results of ABELtrapped Alexa488-Atto594-labeled enzymes. The time traces with donor intensity in blue (I$_D$) and acceptor intensity in green (I$_A$) were binned to 10 ms time intervals. High background count rates had to be subtracted, briefly 138 counts / 10 ms (13.8 kHz) in the donor channel and 41 counts / 10 ms (4.1 kHz) in the acceptor channel. Three types of FRET efficiencies were found for the F$_6$F$_{17}$-ATP synthases in Fig. 3 B, calculated as the proximity factor P=I$_A$/ (I$_D$ + I$_A$). The low FRET efficiency (L) photon bursts originated from enzymes with an actual rotor orientation of ε that was related to a large distance between Atto594 on the ε subunit and Alexa488 on the a subunit. The medium FRET efficiency (M) corresponded to a different rotor orientation, i.e. yielding a shorter distance between the fluorophores. The high FRET efficiency (H) was assigned to a third ε subunit orientation within the enzyme that showed the shortest distance between the two marker dyes. Despite the high luminescence background on both detection channels, ABELtrapping of a single FRET-labeled F$_6$F$_{17}$-ATP synthase in a liposome in buffer was possible when we trapped the biomolecules on the FRET acceptor signal that showed a slightly reduced background. We added 0.1 % PVP to the buffer to prevent sticking of the proteoliposomes on the glass and PDMS surfaces of the chip.

4. DISCUSSION

Here we presented the current version of our ABELtrap setup in Jena based on two fast EOBDs to generate a laser focus pattern in a 2 μm x 2 μm region with up to 66 kHz repetition rate. Adapting the ABELtrap software for the FPGA by A. Fields and A. E. Cohen$^{59}$, we could achieve trapping of single FRET-labeled F$_6$F$_{17}$-ATP synthases in a liposome in buffer solution. Thereby, difficulties with surface-attachment of this nanomotor could be avoided: In future, the function of the enzyme can be studied for extended observation times in such an ABELtrap. In contrast to our previous smFRET approaches with F$_6$F$_{17}$-ATP synthases using freely diffusing proteoliposomes, the fluorescence intensities of donor and acceptor dye on a single enzyme could be analyzed quantitatively, and photophysical fluctuations could be identified helping to exclude these photon bursts from further FRET analysis.

Our previous versions of the ABELtrap, EMCCD-based$^{52}$ or AOBDbased$^{52,80,81}$, respectively, did not reach residence times of more than 100 seconds for fluorescent nanobeads as we have achieved now. However, the actual versions of the ABELtraps in Stanford and in Harvard have been developed even further and have implemented sophisticated learning algorithms for the diffusion and electromobility properties of a trapped molecule. This results in significantly tighter trapping and gains additional information about the hydrodynamic radius and charge (and transient changes thereof) of the trapped molecule.

PDMS/glass ABELtrap chips exhibit strong luminescence upon laser excitation with 491 nm. Therefore, quartz microfluidics are the better choice for the fast photon-by-photon feedback of the current versions of the ABELtrap. In addition, photophysical properties of both FRET donor and acceptor dyes in the respective local protein environment have to be examined carefully, and, eventually, oxygen scavenger systems have to be added to prevent early photobleaching and shortened observation times. All these methods have already been invented, optimized and applied by the ABELtrap groups of A. E. Cohen and W. E. Moerner. Thus we can expect that we will learn more und in detail about the mechanochemical mechanisms of this rotary nanomachine by observing it one after another in real time and at work in the ABELtrap.

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REFERENCES

[1] Borsch, M., Turina, P., Eggeling, C., Fries, J. R., Seidel, C. A., Labahn, A. and Graber, P., "Conformational changes of the H+-ATPase from Escherichia coli upon nucleotide binding detected by single molecule fluorescence," FEBS letters 437, 251-254 (1998)

[2] Steinbrecher, T., Hucke, O., Steigmiller, S., Borsch, M. and Labahn, A., "Binding affinities and protein ligand complex geometries of nucleotides at the F(1) part of the mitochondrial ATP synthase obtained by ligand docking calculations," FEBS letters 530, 99-103 (2002)

[3] Borsch, M., Diez, M., Zimmermann, B., Reuter, R. and Graber, P., "Monitoring gamma-subunit movement in reconstituted single EFoF1 ATP synthase by fluorescence resonance energy transfer," in Fluorescence spectroscopy, Imaging and Probes. New Tools in Chemical, Physical and Life Sciences Kraayenhof, R., Visser, A. J. W. and Gerritsen, H. C., Eds., pp. 197-207, Springer-Verlag, Berlin (2002)

[4] Borsch, M., Diez, M., Zimmermann, B., Trost, M., Steigmiller, S. and Graber, P., "Stepwise rotation of the gamma-subunit of EFoF1-ATP synthase during ATP synthesis: a single-molecule FRET approach," Proc. SPIE 4962, 11-21 (2003)

[5] Diez, M., Zimmermann, B., Borsch, M., Konig, M., Schweinberger, E., Steigmiller, S., Reuter, R., Felekyan, S., Kudryavtsev, V., Seidel, C. A. and Graber, P., "Proton-powered subunit rotation in single membrane-bound FoF1-ATP synthase," Nature structural & molecular biology 11, 135-141 (2004)

[6] Boldt, F. M., Heinez, J., Diez, M., Petersen, J. and Borsch, M., "Real-time pH microscopy down to the molecular level by combined scanning electrochemical microscopy/single-molecule fluorescence spectroscopy," Anal Chem 76, 3473-3481 (2004)

[7] Steigmiller, S., Zimmermann, B., Diez, M., Borsch, M. and Graber, P., "Binding of single nucleotides to H+-ATP synthases observed by fluorescence resonance energy transfer," Bioelectrochemistry 63, 79-85 (2004)

[8] Diez, M., Borsch, M., Zimmermann, B., Turina, P., Dunn, S. D. and Graber, P., "Binding of the b-subunit in the ATP synthase from Escherichia coli," Biochemistry 43, 1054-1064 (2004)

[9] Krebstakies, T., Zimmermann, B., Graber, P., Altendorf, K., Borsch, M. and Greie, J. C., "Both rotor and stator subunits are necessary for efficient binding of F1 to F0 in functionally assembled Escherichia coli ATP synthase," The Journal of biological chemistry 280, 33338-33345 (2005)

[10] Zimmermann, B., Borsch, M., Zarrabi, N., Graber, P. and Borsch, M., "Movements of the epsilon-subunit during catalysis and activation in single membrane-bound H+-ATP synthase." Embo J 24, 2053-2063 (2005)

[11] Steigmiller, S., Borsch, M., Graber, P. and Huber, M., "Distances between the b-subunits in the tether domain of F0(F1)-ATP synthase from E. coli," Biochimica et biophysica acta 1708, 143-153 (2005)

[12] Zarrabi, N., Zimmermann, B., Diez, M., Graber, P., Wrachtrup, J. and Borsch, M., "Asymmetry of rotational catalysis of single membrane-bound FoF1-ATP synthase," Proc. SPIE 5699, 175-188 (2005)

[13] Zimmermann, B., Diez, M., Borsch, M. and Graber, P., "Subunit movements in membrane-integrated EFoF1 during ATP synthesis detected by single-molecule spectroscopy," Biochimica et biophysica acta 1757, 311-319 (2006)

[14] Zarrabi, N., Duser, M. G., Reuter, R., Dunn, S. D., Wrachtrup, J. and Borsch, M., "Detecting substeps in the rotary motors of FoF1-ATP synthase by Hidden Markov Models," Proc. SPIE 6444, 64440E (2007)

[15] Zarrabi, N., Duser, M. G., Ernst, S., Reuter, R., Glick, G. D., Dunn, S. D., Wrachtrup, J. and Borsch, M., "Monitoring the rotary motors of single FoF1-ATP synthase by synchronized multi channel TCSPC," Proc. SPIE 6771, 67710F (2007)

[16] Peneva, K., Mihov, G., Herrmann, A., Zarrabi, N., Borsch, M., Duncan, T. M. and Mullen, K., "Exploiting the Nitrilotriacetic Acid Moiety for Biolabeling with Ultrastable Perylene Dyes," Journal of the American Chemical Society 130, 5398-5399 (2008)

[17] Diepholz, B., Borsch, M. and Bottcher, B., "Structural organization of the V-ATPase and its implications for regulatory assembly and disassembly," Biochemical Society transactions 36, 1027-1031 (2008)

[18] Galvez, E., Duser, M., Borsch, M., Wrachtrup, J. and Graber, P., "Quantum dots for single-pair fluorescence resonance energy transfer in membrane- integrated EfoF1," Biochemical Society Transactions 36, 1017-1021 (2008)

[19] Duser, M. G., Bi, Y., Zarrabi, N., Dunn, S. D. and Borsch, M., "The proton-translocating a subunit of FoF1-ATP synthase is allocated asymetrically to the peripheral stalk," The Journal of biological chemistry 283, 33602-33610 (2008)

[20] Zarrabi, N., Ernst, S., Duser, M. G., Golovina-Leiker, A., Becker, W., Erdmann, R., Dunn, S. D. and Borsch, M., "Simultaneous monitoring of the two coupled motors of a single FoF1-ATP synthase by three-color FRET using duty cycle-optimized triple-ALEX," Proc. SPIE 7185, 718505 (2009)

[21] Johnson, K. M., Swenson, L., Oppari, A. W., Jr., Reuter, R., Zarrabi, N., Fierke, C. A., Borsch, M. and Glick, G. D., "Mechanistic basis for differential inhibition of the F(1)F(o)-ATPase by auromycin," Biopolymers 91, 830-840 (2009)

[22] Duser, M. G., Zarrabi, N., Cipriano, D. J., Ernst, S., Glick, G. D., Dunn, S. D. and Borsch, M., "36 degrees step size of proton-driven c-ring rotation in FoF1-ATP synthase," Embo J 28, 2689-2696 (2009)

[23] Modesti, G., Zimmermann, B., Borsch, M., Herrmann, A. and Saalwachter, K., "Diffusion in Model Networks as Studied by NMR and Fluorescence Correlation Spectroscopy," Macromolecules 42, 4681-4689 (2009)

[24] Borsch, M., "Single-molecule fluorescence resonance energy transfer techniques on rotary ATP synthases," Biological chemistry 392, 135-142 (2011)

[25] Borsch, M. and Wrachtrup, J., "Improving FRET-based monitoring of single chemomechanical rotary motors at work," Chemphyschem 12, 542-553 (2011)

[26] Seyer, F., Oosaka, T., Yagimura, H., Ernst, S., Noji, H., Lino, R. and Borsch, M., "Subunit rotation in a single F1[sub o]F1[sub 1]-ATP synthase in a living bacterium monitored by FRET," Proc. SPIE 7905, 79050K (2011)
[27] Ernst, S., Duser, M. G., Zarrabi, N. and Borsch, M., "Three-color Förster resonance energy transfer within single FoF1-ATP synthases: monitoring elastic deformations of the rotary double motor in real time," *J Biomed Opt* 17, 011004 (2012)

[28] Ernst, S., Duser, M. G., Zarrabi, N., Dunn, S. D. and Borsch, M., "Elastic deformations of the rotary double motor of single FoF1-ATP synthases detected in real time by Förster resonance energy transfer," *Biochimica et Biophysica Acta (BBA) - Bioenergetics* 1817, 1722-1731 (2012)

[29] Hammann, E., Zappe, A., Keis, S., Ernst, S., Matthies, D., Meier, T., Cook, G. M. and Borsch, M., "Step size of the rotary proton motor in single FoF1-ATP synthase from a thermoalkaliphilic bacterium by DCO-ALEX FRET," *Proc. SPIE* 8228, 82280A (2012)

[30] Sielaff, H. and Borsch, M., "Twisting and subunit rotation in single FOF1-ATP synthase," *Phil Trans R Soc B* 368, 20120024 (2013)

[31] Borsch, M., "Microscopy of single FoF1-ATP synthases— The unraveling of motors, gears, and controls," *IUBMB life* 65, 227-237 (2013)

[32] Heitkamp, T., Sielaff, H., Korn, A., Renz, M., Zarrabi, N. and Borsch, M., "Monitoring subunit rotation in single FRET-labeled FoF1-ATP synthase in an anti-Brownian electrokinetic trap," *Proc. SPIE* 8588, 85880Q (2013)

[33] Borsch, M. and Duncan, T. M., "Spotlighting motors and controls of single FoF1-ATP synthase," *Biochemical Society transactions* 41, 1219-1226 (2013)

[34] Bockenhauer, S. D., Duncan, T. M., Moermer, W. E. and Borsch, M., "The regulatory switch of F1-ATPase studied by single-molecule FRET in the ABEL Trap," *Proc. SPIE* 8950, 89500H (2014)

[35] Boyer, P. D., "The ATP synthase—a splendid molecular machine," *Annu Rev Biochem* 66, 717-749 (1997)

[36] Yoshida, M., Muneyuki, E. and Hisabori, T., "ATP synthase - a marvellous rotary engine of the cell," *Nat Rev Mol Cell Biol* 2, 669-677 (2001)

[37] Abrahamsson, J. P., Leslie, A. G., Lutter, R. and Walker, J. E., "Structure at 2.8 Å resolution of F1-ATPase from bovine heart mitochondria," *Nature* 370, 621-628 (1994)

[38] von Ballmoos, C., Cook, G. M. and Dimroth, P., "Unique rotary ATP synthase and its biological diversity," *Annual review of biophysics* 37, 43-64 (2008)

[39] Armbruster, A., Hohn, C., Hermesdorf, A., Schumacher, K., Borsch, M. and Gruber, G., "Evidence for major structural changes in subunit C of the vacuolar ATPase due to nucleotide binding," *FEBS letters* 579, 1961-1967 (2005)

[40] Borsch, M., "Targeting cytochrome C oxidase in mitochondria with Pt(II)-labeled ALEX FRET," *Proc. SPIE* 7569, 75690W (2011)

[41] Zarrabi, N., Heitkamp, T., Greie, J.-C. and Borsch, M., "Monitoring the conformational dynamics of a single potassium transporter by ALEX-FRET," *Proc. SPIE* 6862, 68620M (2008)

[42] Alemdaroglu, F. E., Alexander, S. C., Ji, D. M., Prusty, D. K., Borsch, M. and Herrmann, A., "Poly(BODIPY)s: A New Class of Tunable Polymeric Dyes," *Macromolecules* 42, 6529-6536 (2009)

[43] Winnewisser, C., Schneider, J., Borsch, M. and Rotter, H. W., "In situ temperature measurements via ruby R lines of sapphire substrate based InGaN light emitting diodes during operation," *Journal of Applied Physics* 89, 3091-3094 (2001)

[44] Tisler, J., Balasubramanian, G., Naydenov, B., Kolesov, R., Grotz, B., Reuter, R., Boudou, J. P., Curni, P. A., Sennour, M., Thorel, A., Borsch, M., Aulenbacher, K., Erdmann, R., Hemmer, P. R., Jelezko, F. and Wrachtrup, J., "Fluorescence and Spin Properties of Defects in Single Digit Nanodiamonds," *ACS nano* 3, 1959-1965 (2009)

[45] Heitkamp, T., Sielaff, H., Korn, A., Renz, M., Zarrabi, N. and Borsch, M., "Drug transport mechanism of P1A1O1 ATP synthase subunit B from Methanosarcina mazei Go1: Implications of nucleotide-binding differences in the major P1A1O1 subunits A and B," *Journal of molecular biology* 358, 725-740 (2006)

[46] Armbruster, A., Hohn, C., Hermesdorf, A., Schumacher, K., Borsch, M. and Gruber, G., "Evidence for major structural changes in subunit C of the vacuolar ATPase due to nucleotide binding," *FEBS letters* 579, 1961-1967 (2005)

[47] Borsch, M., "Targeting cytochrome C oxidase in mitochondria with Pt(II)-porphyrins for photodynamic therapy," *Proc. SPIE* 7551, 75510G (2010)

[48] Ernst, S., Schonbauer, A. K., Bar, G., Borsch, M. and Kuhn, A., "YidC-driven membrane insertion of single fluorescent Pf3 coat proteins," *Journal of molecular biology* 412, 165-175 (2011)

[49] Ernst, S., Verhalen, B., Zarrabi, N., Wilkens, S. and Borsch, M., "Drug molecule fluorescence resonance energy transfer," *Proc. SPIE* 7003, 790328 (2011)

[50] Verhalen, B., Ernst, S., Borsch, M. and Wilkens, S., "Dynamic ligand induced conformational rearrangements in P-glycoprotein as probed by fluorescence resonance energy transfer spectroscopy," *Journal of Biological Chemistry* 287, 1112-1127 (2012)

[51] Winterfeld, S., Ernst, S., Borsch, M., Gerken, U. and Kuhn, A., "Real time observation of single membrane protein insertion events by the Escherichia coli insertase YidC," *Plos one* 8, e59023 (2013)

[52] Zarrabi, N., Ernst, S., Verhalen, B., Wilkens, S. and Borsch, M., "Analyzing conformational dynamics of single P-glycoprotein transporters by Förster resonance energy transfer using hidden Markov models," *Methods* 66, 168-179 (2014)

[53] Yasuda, R., Noji, H., Yoshida, M., Kinosita, K., Jr. and Itoh, H., "Resolution of distinct rotational substeps by submillisecond kinetic analysis of F1-ATPase," *Nature* 410, 898-904 (2001)

[54] Bilyard, T., Nakashishi-Matsui, M., Stein, B. C., Pilizota, T., Nord, A. L., Hosokawa, H., Futai, M. and Berry, R. M., "High-resolution single-molecule characterization of the enzymatic states in Escherichia coli F1-ATPase," *Philosophical Transactions of the Royal Society B: Biological Sciences* 368, 20120023 (2013)
[55] Suzuki, T., Tanaka, K., Wakabayashi, C., Saita, E. and Yoshida, M., "Chemomechanical coupling of human mitochondrial F1-ATPase motor," *Nature chemical biology* 10, 930-936 (2014)

[56] Cohen, A. E. and Moerner, W. E., "The anti-Brownian electrophoretic trap (ABEL trap): fabrication and software," *Proc. SPIE* 5699, 296-305 (2005)

[57] Cohen, A. E. and Moerner, W. E., "An all-glass microfluidic cell for the ABEL trap: fabrication and modeling," *Proc. SPIE* 5930, 59300S (2005)

[58] Cohen, A. E. and Moerner, W. E., "Method for trapping and manipulating nanoscale objects in solution," *Appl. Phys. Lett.* 86, 093109 (2005)

[59] Cohen, A. E. and Moerner, W. E., "Principal-components analysis of shape fluctuations of single DNA molecules," *Proceedings of the National Academy of Sciences of the United States of America* 104, 12622-12627 (2007)

[60] Cohen, A. E. and Moerner, W. E., "Internal mechanical response of a polymer in solution," *Phys Rev Lett* 98, 116001 (2007)

[61] Cohen, A. E. and Moerner, W. E., "Controlling Brownian motion of single protein molecules and single fluorophores in aqueous buffer," *Optics express* 16, 6941-6956 (2008)

[62] Jiang, Y., Wang, Q., Cohen, A. E., Douglas, N., Frydman, J. and Moerner, W. E., "Hardware-based anti-Brownian electrokinetic trap (ABEL trap) for single molecules: control loop simulations and application to ATP binding stoichiometry in multi-subunit enzymes," *Proc. SPIE* 7038, 703807 (2008)

[63] Goldsmith, R. H. and Moerner, W. E., "Watching conformational- and photodynamics of single fluorescent proteins in solution," *Nature chemistry* 2, 179-186 (2010)

[64] Wang, Q. and Moerner, W. E., "Optimal strategy for trapping single fluorescent molecules in solution using the ABEL trap," *Appl Phys B* 99, 23-30 (2010)

[65] Goldsmith, R. H. and Moerner, W. E., "Watching conformational- and photo-dynamics of single fluorescent proteins in solution," *Nature chemistry* 2, 179-186 (2010)

[66] Bockenhauer, S., Furstenberg, A., Yao, X. J., Kobila, B. K. and Moerner, W. E., "Conformational dynamics of single G protein-coupled receptors in solution," *J Phys Chem B* 115, 13328-13338 (2011)

[67] Wang, Q. and Moerner, W. E., "An Adaptive Anti-Brownian E lectrokinetic trap with real-time information on single-molecule diffusivity and mobility." *ACS nano* 5, 5792-5799 (2011)

[68] Jiang, Y., Douglas, N. R., Conley, N. R., Miller, E. J., Frydman, J. and Moerner, W. E., "Sensing cooperativity in ATP hydrolysis for single multisubunit enzymes in solution," *Proceedings of the National Academy of Sciences of the United States of America* 108, 16962-16967 (2011)

[69] Wang, Q., Goldsmith, R. H., Jiang, Y., Bockenhauer, S. D. and Moerner, W. E., "Probing single biomolecules in solution using the anti-Brownian electrokinetic (ABEL) trap," *Acc Chem Res* 45, 1955-1964 (2012)

[70] Bockenhauer, S. D., Wang, Q. and Moerner, W. E., "Spectrally resolved anti-Brownian electrokinetic (ABEL) trapping of single peridinin-chlorophyll-proteins in solution," *Proc. SPIE* 8427, 84274C (2012)

[71] Thompson, M. A., Lew, M. D. and Moerner, W. E., "Extending microscopic resolution with single-molecule imaging and active control," *Annual review of biophysics* 41, 321-342 (2012)

[72] Wang, Q. and Moerner, W. E., "Lifetime and Spectrally Resolved Characterization of the Photodynamics of Single Fluorophores in Solution Using the Anti-Brownian Electrokinetic Trap," *J Phys Chem B* 117, 4641-4648 (2013)

[73] Schlau-Cohen, G. S., Wang, Q., Southall, J., Cogdell, R. J. and Moerner, W. E., "Single-molecule spectroscopy reveals photosynthetic LH2 complexes switch between emissive states," *Proceedings of the National Academy of Sciences of the United States of America* 110, 10899-10903 (2013)

[74] Bockenhauer, S. D. and Moerner, W. E., "Photo-induced conformational flexibility in single solution-phase peridinin-chlorophyll-proteins," *The journal of physical chemistry* 117, 8399-8406 (2013)

[75] Wang, Q. and Moerner, W. E., "Single-molecule motions enable direct visualization of biomolecular interactions in solution," *Nature methods* 11, 555-558 (2014)

[76] Fields, A. P. and Cohen, A. E., "Anti-Brownian traps for studies on single molecules," *Methods Enzymol* 475, 149-174 (2010)

[77] Cohen, A. E. and Fields, A. P., "The cat that caught the canary: what to do with single-molecule trapping," *ACS nano* 5, 5296-5299 (2011)

[78] Cohen, A. E., "Control of nanoparticles with arbitrary two-dimensional force fields," *Phys Rev Lett* 94, 118102 (2005)

[79] Fields, A. P. and Cohen, A. E., "Electrokinetic trapping at the one nanometer limit," *Proceedings of the National Academy of Sciences of the United States of America* 108, 8937-8942 (2011)

[80] Zarrabi, N., Clausen, C., Duser, M. G. and Borsch, M., "Manipulating freely diffusing single 20-nm particles in an Anti-Brownian Electrokinetic Trap (ABELtrap)," *Proc. SPIE* 8587, 85870L (2013)

[81] Sielaff, H., Heitkamp, T., Zappe, A., Zarrabi, N. and Borsch, M., "Subunit rotation in single FRET-labeled F1-ATPase hold in solution by an anti-Brownian electrokinetic trap," *Proc. SPIE* 8590, 859008 (2013)

[82] Rendler, T., Renz, M., Hammann, E., Ernst, S., Zarrabi, N. and Borsch, M., "Monitoring single membrane protein dynamics in a liposome manipulated in solution by the ABELtrap," *Proc. SPIE* 7902, 79020M (2011)