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Feedback controlled electro-kinetic traps for single molecule spectroscopy

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Abstract. One of the principal limitations of single-molecule spectroscopy in solution is the diffusion-limited residence time of a given molecule within the detection volume. A common solution to this problem is to immobilize molecules of interest on a passivated glass surface for extending the observation time for obtaining reliable data statistics. However, surface tethering of molecules often introduces artifacts, particularly when studying the structural dynamics of biomolecules. To circumvent this limitation, we investigated alternative ways to extend single-molecule observation times in solution without surface immobilization. Among various possibilities, the so-called Anti-Brownian electro-kinetic trap (or ABEL trap) seems best suited to achieve this goal. The essential part of that trap is a feedback-controlled electro-kinetic steering of a molecule's position in reaction to its diffusive Brownian motion which is monitored by fluorescence, thus keeping the molecule within a sub-micron sized detection volume. Fluorescence trace recording of over thousands of milliseconds duration on individual dye molecules within an ABEL trap have been reported. In this short review, we will briefly discuss the principle and some results of ABEL-trapping of individual molecules with possible extensions to future works.

Keywords. Single molecule fluorescence spectroscopy, active trapping of molecules, Anti-Brownian Electrophoretic Trap

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1. Introduction

The ultimate goal when studying the wonders of nature and its processes at the molecular level is to be able to detect and investigate processes and structures at the level of single molecules. In the past four decades, advances in laser sources, opto-electric detectors, and high-performance optical microscopes, have made single-molecule fluorescence spectroscopy and imaging a routine method in many labs around the world. Nowadays, single-molecule spectroscopy is widely used for detecting and monitoring the properties

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of individual molecules in various systems and environments such as in solids and polymers, on surfaces, or in liquids. Arguably, single-molecule spectroscopy in a fluid environment has drawn most of the attention of the research community which is interested in studying biophysical or biological systems under ambient and physiological conditions. The potentials and prospects of room temperature single-molecule spectroscopy (SMS) in biological systems have been covered in numerous reviews [1–13] and books [14–17].

Over the years, optical studies of single molecules have become a method which is capable of addressing many intriguing questions in much greater detail than ensemble spectroscopy. Many of the initial applications of SMS in biology have been in the area of extremely sensitive imaging and analyte detection. While these applications will surely continue to be important also in the future, there is a large number of recent studies that are using SMS for investigating the mechanisms of protein folding/unfolding, biomolecular interactions, and the relation between structure and function of biomolecules. For example, a common aspect of protein folding (or misfolding) is the large degree of structural or configurational heterogeneity during structure formation, and partially also of the final structures. Although ensemble experiments have provided a wealth of experimental data on protein folding, they can only yield restricted information on the microscopic folding pathways and mechanisms. If one considers, for example, a simple two-state folder, bulk measurements will always yield only an average of the ensemble molecular behavior, which is a superposition of the already-folded and the still-unfolded states. The fast dynamics between conformers in the unfolded and folded state will be invisible to the bulk measurement. In contrast to ensemble measurements, SMS promises direct access to information on individual folding pathways, as well as to the internal dynamics between conformers in the unfolded and folded state [9, 18–24].

2. What are the restrictions of current single-molecule detection techniques?

There are several ways of performing single molecule spectroscopy. One is fluorescence correlation spectroscopy (FCS), which is a powerful and versatile technique for measuring the diffusion, concentration, and fast dynamical processes with single-molecule sensitivity [25–27]. A general schematic for single molecule detection and FCS is shown in Figure 1. FCS was originally introduced by Elson, Magde and Webb [28–30] in the early seventies and has become a very popular technique over the last two decades. This was facilitated by the development of high-quality objectives having large numerical apertures, the wide distribution of affordable laser sources, and the introduction of solid-state single-photon detectors with better than 50% quantum efficiency. Today, FCS has become an important spectroscopic technique that is used in numerous biophysical and physicochemical studies [27, 31–43]. In FCS, one records the fluorescence signal generated by a focused laser beam within a tiny volume in a sample and evaluates the signal fluctuations due to the diffusion and/or interaction or conformational dynamics of molecules which diffuse trough the observation volume. Although the method requires true single-molecule sensitivity, it is an accumulating technique which sums up a fluorescence correlation function over the transits of many single molecules. Thus, it can not reveal the structure or dynamics of *one* single molecule per se.

An alternative method of SMS is to really identify single-molecule signals when they transit through the detection volume, and to evaluate these signals in a molecule-by-molecule fashion. This has become particularly popular with the advent of single-molecule Fŕster resonance energy transfer [9, 44–48]. Meanwhile, sophisticated methods of data evaluation have been worked out to extract a maximum of information from such single-

molecule measurements [49–61].

However, a limitation of all these techniques which are applicable to freely diffusing molecules in solution is the short observation time for individual molecules (time during which a signal from one and the same molecule can be measured), which lasts only a few 100 microseconds up to a few milliseconds. This is due to the thermal Brownian motion of molecules in solution and the smallness of typical detection volumes in SMS systems, which are on the order of one femtoliter. This limits the number of detectable fluorescence photons from one and the same molecule and thus prevents the precise determination of any spectroscopic parameters on a single-molecule level, but also the observation of any dynamical processes which last longer than the observation time. Consequently, when for example studying the dynamics of single proteins, one has to accumulate signal over many molecule transits and thus "averages" in effect the process one wants to observe. In principle, one could increase the diffusion-limited residence time by increasing the observation volume, but such a strategy dramatically lowers the signal-to-background ratio.

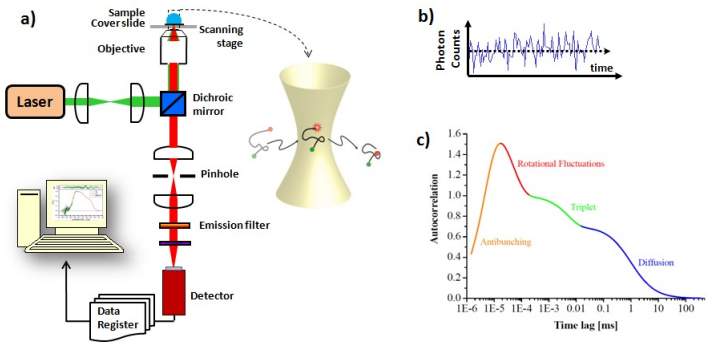

Figure 1. A simple schematic of a single-molecule detection setup for either freely diffusing molecules in solution or for molecules immobilized on surfaces (a). FCS, which comes in the first category, is used to study both molecular diffusion as well as equilibrium kinetics of chemical systems by monitoring fluctuations in fluorescence intensity (and thus concentration) coming from a small detection volume. Any process that leads to a fluctuating intensity (b) can be followed by FCS, and it achieves highest sensitivity when there is, on average, only one molecule within the confocal observation volume. A typical autocorrelation function from picoseconds to seconds is shown in panel (c). The temporal decay of the autocorrelation function is defined by diffusion, and its variance provides the average number of fluorescent molecules in the detection volume. A typical FCS measurement provides information about diffusion coefficient, photophysical rate constants, and sample concentrations. In addition, aggregation processes and rotational diffusion can also be studied in this manner.

In order to extend the diffusion-limited residence time of a molecule within the detection volume, various concepts have been considered. One approach uses immobilization of molecules on a surface by covalent/non-covalent attachment, or by including them within the pores of a gel, see. e.g. [62]. But when using such immobilization strategies, there is no guarantee that one does not significantly change the dynamics and structures of the molecules one wants to investigate, in comparison to their dynamics and structure in solution. A better alternative, especially for biophysical/biological applications, is surface tethering where one introduces an additional spacer between the surface and the molecule.

But again, one has to check from case to case how much this influences the process one wants to study. An even better approach is to encapsulate a molecule into an immobilized nano-container (i.e. a lipid vesicle) [63–65]. However, such an approach is tedious, time consuming, and does not easily allow for changing the liquid environment of molecule, or to bring it in contact with other molecules.

Although SMS so far has resulted in many scientific advances, it would be ideal if one could combine the advantages of both techniques, namely by watching single molecules for long observation times without perturbing them with surface attachment. Hence, one of the key challenges in advanced single molecule spectroscopy, especially in biophysics, is to develop methods for trapping and manipulating individual molecules within their native environment without any attachment.

3. Alternative possibilities

An alternative to covalent/non-covalent attachment to surfaces or nano-encapsulation are the employment of optical tweezers [66]. Optical tweezers have led to a revolution in the nano-manipulation of micron-sized objects. Unfortunately, the forces generated by optical tweezers scale with the volume of the trapped objects and are much too weak to trap objects smaller than roughly 10 nm in solution and at room temperature. To trap a 10 nm object, it requires a million times more laser power than trapping an object of one micrometer, as the force generated is proportional to the object's electric polarizability, which in turn is proportional to its volume. Such intense light runs the risk of destroying the analyte rather than trapping it. Moreover, the trapping force arises through a second-order interaction between the gradient of the electromagnetic field and the induced dipole.

In the early eighties, Howard Berg studied the motion of single bacteria under a microscope with camera-based detection [67, 68]. He came to the idea to keep the moving bacteria in focus and within the field of view by actively moving the sample stage in feedback to the bacterial motion. After a measurement row, he could then reconstruct the motion of a bacterium by evaluating the performed adjustments steps of the microscope's stage. However, the temporal and spatial resolution of this method is not sufficient to apply it to the tracking of single small molecules in solution.

Inspired by the work of Howard Berg, one of us (J.E,) proposed to combine the high speed of point-detectors with a quasi-imaging approach by rapidly moving the detection region in space and thus to obtain a quasi-simultaneous scan-image of a small region in space [69, 70]. This allows determining the position and motion of individual molecules and by using a clever feedback algorithm to follow molecules along the diffusion path. This concept was theoretically studied for the tracking of single molecules moving within a plane, such as an artificial lipid layer or a cell membrane. The idea was to use the feedback to translate either the laser focus or the microscope stage to keep a diffusing fluorescent molecule within the focal volume of the confocal microscope, cf. Figure 2. Variants of this scheme have later been implemented experimentally by different groups, and there is continuing theoretical interest in developing optimal strategies for tracking diffusing molecules [71–77]. The core limitation of this approach is the limited response time and travel-length of the mechanical translation mechanism which is used for moving the focus in synchrony with the diffusing particle, which allows its application only to tracking objects with moderate diffusion speed.

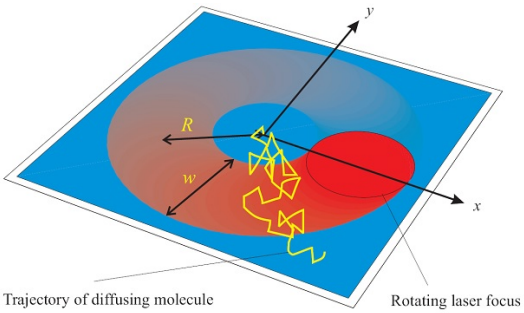

Figure 2. General scheme of the proposed experimental set-up by Enderlein. A Gaussian laser beam is tightly focused into the plane of diffusion of a molecule to be observed. The laser focus is moved around a circle with radius R. After each rotation, the center of the circle of rotation is adjusted to a new position according to the photon detection intensities observed along the rotation.

4. The Anti-Brownian Electrophoretic Trap

Recently, an extension of the idea of actively tracking a diffusing molecule which can provide active trapping for nanometer-sized objects for long observation time without thermal load or immobilization has been developed by Moerner and Cohen with their so-called Anti-Brownian Elektrokinetic or ABEL trap [78–87]. The only requirement of their approach is that the trapped object can be optically imaged. Instead of mechanically shifting the focal spot through the sample, the ABEL trap employs electroosmosis for moving a molecule back into focus. Electroosomosis are commonly used to separate biomolecules. Due to the first order interaction, the force generated on molecules with the ABEL trap is much stronger than that achieved by optical tweezers and also depends on the linear size of the analyte molecule rather than its volume.

The essential idea of the ABEL trap is the combination of fluorescence-based position estimation with a fast electro-kinetic feedback to exactly counter the Brownian motion of single nanoscale objects. Feedback control has often been used to counteract thermal noise in stochastic systems, similar to the case of the ABEL trap where the feedback is used to cancel the Brownian motion of single nanoscale objects in solution, over some finite bandwidth. Objects as small as 15 nm in diameter can be held in feedback traps with less than 1 mW of laser power (required solely for imaging the position of the object).

5. How tightly does an ABEL trap confine a particle?

In a feedback controlled ABEL trap, one uses confocal microscopy to track the position of a molecule by detecting the fluorescence signal, and applies a feedback-controlled displacement to keep the molecule close to a target location. A schematic is shown in Figure 3. The bottlenecks in the feedback controlled ABEL trap are the feedback latency and the achievable photon detection rate. Other important factors are the accuracy of position estimation and the strength of the restoring force.

Between iterations of the feedback loop, a molecule may diffuse a distance (Δx) away

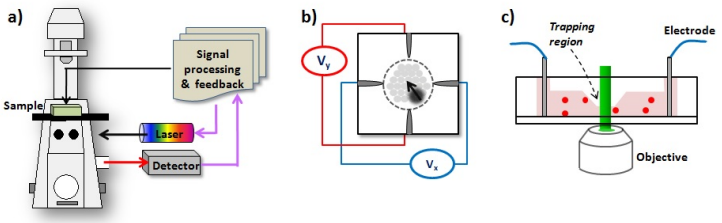

Figure 3. General layout of the feedback controlled electro-kinetic trap (ABEL trap) (a). The signal processing and feedback hardware controls and registers detector signal for precise determination of position and applies feedback forces to compensate for the Brownian motion. For applying voltages, the disc-shaped trapping region in the microfluidic cell is connected to four deep control-electrode channels in the x-y-plane. Top view of the trapping cell, with 27 laser spots in a hexagonal lattice pattern is shown in the center (b). The dashed circle indicates the trapping region. Any fluorophore (dark circle) undergoing Brownian motion is displaced back to the center of the trap with countering electro-kinetic forces. (c) Side view of the microfluidic cell assembly.

from the target location, while the length of this distance depends on the diffusion coefficient (D) of the particle and the latency of the feedback (τ), $\Delta x \approx \sqrt{2D\tau}$. This distance should be much smaller than the dimension of the observation volume; otherwise large thermal fluctuations would knock the particle completely out of the trapping region. Hence, the principal criterion for the feedback control is to have a feedback latency which is significantly smaller that the square of the size of the trap region divided by two times the diffusion coefficient. Even with perfect feedback hardware, the feedback latency is limited by the finite photon detection-rate, i.e. how many photons per unit of time can be squeezed out from the trapped object. Moreover, blinking of fluorophores (with long off-times) and the presence of long-living triplet states render the molecule untraceable and it may be eventually lost from the trap. Photobleaching is another limitation, which can, however, be improved by adding various chemicals to the solution, i.e. triplet state quenchers or oxygen scavengers [88, 89].

For localizing a single molecule with sufficient accuracy, one needs some minimum number of detected photons for localizing a single molecule with sufficient accuracy. Moreover, the position of a single molecule gets blurred over time due to its diffusion. Hence, one should be cautious in optimizing the system parameters in such a manner that the decrease in uncertainty due to signal accumulation is not outweighed by the increase in uncertainty due to diffusion. Furthermore, attention should also be paid to the perfection of the optical design, and to the purity of chemicals/solvents which is necessary to minimize spurious signals which can reduce tracking performance. Trapping can be done in most standard buffers.

In the ABEL trap, electrokinetic forces are so strong that the trapping efficiency is limited by the latency of the feedback loop. The effective spring constant of the trap is given by $k = k_B T/\Delta x^2$, while the mean-square amplitude for position fluctuations is $2D\tau$. As D is inversely proportional to the radius r of the fluorophore, the effective spring constant for ABEL trapping is directly proportional to the fluorophore's radius

(i.e. $k \propto r$). In comparison, for an optical trap the effective spring constant scales as $k \propto r^3$. Consequently, the ABEL trap is much better suited for trapping smaller objects than an optical trap. It should be noted that the feedback forces in an ABEL trap are applied as body forces to all molecules in the solution and is thus perfectly suited to the non-perturbative observation of single-molecules.

6. Essentials for building an electro-kinetic trap

The heart of the ABEL trap is the microfluidic glass cell, which is prepared following a series of photolithography and dry and wet etching procedures. The diced wafers are then chemically bonded to a thin cover slide, and an appropriate PDMS mask has been designed to cover the diced wafer on the cover slide and also to introduce the two pairs of counter electrodes. A schematic diagram of the microfluidic cell is shown in Figure 4.

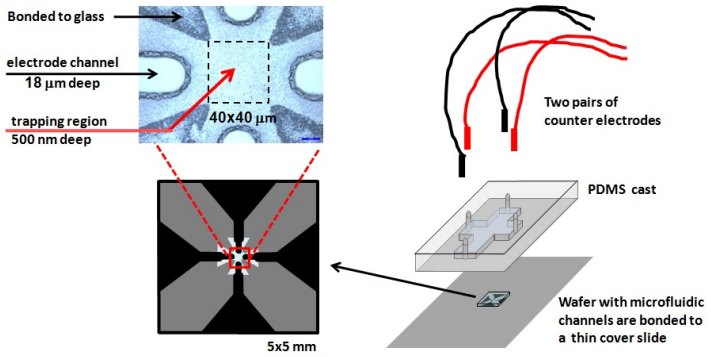

Figure 4. Microfluidic channels and schematic of the chip layout. The fused silica sample cell contains a central trapping region of 500 nm depth connected to deeper channels of 18 micrometer depth. The etched 5 mm square piece of fused silica is bonded to a thin 25 mm square fused-silica cover-slide by silicate bonding at 90°C. A cast piece of poly-dimethylsiloxane, PDMS, is reversibly bonded to the cover slide to contain the fluid and to hold the platinum electrodes in place.

Fluorophores within the pancake-shaped (ca. 60 micrometer wide and ca. 500 nm high) trapping region in a microfluidic cell are confined to the focal plane of the microscope but are free to diffuse within the lateral plane. This microfluidic cell is mounted on the observation stage of an inverted fluorescence microscope. Two pairs of fluidic channels (ca. 20 micron deep) convey voltages from macroscopic control electrodes to the corners of the trapping region (cf. Figure 4). Application of voltage $\mathbf{V} = (V_x, V_y)$ to the control electrodes leads to a force $\mathbf{F} \propto \mathbf{V}$ on the fluorophore in the trapping region. The origin of this force can be of electrophoretic and electroosmotic in nature. Charged particles are directly pulled by the electric field, resulting in an electrophoretic drift. But the applied electric field leads also to an electroosmotic flow in the trapping region, which imparts a hydrodynamic force on all molecules. As in capillary electrophoresis, the relative contributions of these two mechanisms may be adjusted by tailoring the composition of the solution and the surface chemistry of the microfluidic channels. For strongly charged analytes, one generally wants to minimize electroosmotic flow so that the measured mobility reflects intrinsic properties of the particle (i.e. its zeta-potential). On the other hand, for neutral analytes, electroosmosis provides the only way of applying a feedback, which

requires a strong electroosmotic flow.

A broadband super-continuum laser with an acousto-optical tunable filter (AOTF) is the optimal excitation source due the free choice of the excitation wavelength. Two electro-optic deflectors (EODs) are used to steer the position of the beam within the focal plane of the objective. EODs allow much faster beam deflection without any wavelength dispersion, in contrast to acousto-optic deflectors (AODs) [90]. The optical system is designed in such way that it focuses the excitation light into a diffraction-limited spot and to convert the EOD deflections into pure translations of the beam in the focal plane. The emitted light is collected by the same objective and then focused through a pinhole for confocal detection and imaged onto the APDs. For studying fluorescence anisotropy or FRET, one will need two to four detectors. Amongst various focus scan patterns, the hexagonal lattice maximizes the uniformity of the time-averaged illumination while also maximizing the sensitivity of the tracking system to small displacements of the particle or molecule. With fast EODs and a fieldprogrammable gate array (FPGA), it is possible to trap single dye molecules with conventional laser irradiation powers. A general schematic of the setup is shown in Figure 5.

The key to the trap's performance is a statistically rigorous hardware-based real-time tracking and feedback system, implemented on the FPGA. The FPGA records the position of the laser beam at the time of the APD pulse and the positions of the photons detected in one scan cycle are averaged to form a raw measurement of the position of the particle. This system operates at the quantum-limit imposed by the finite information carried by each fluorescence photon. A more sophisticated approach by incorporating a Kalman filter (a "running average" in which recently detected photons are weighted more heavily than those detected earlier) into the FPGA allows for operating the tracking system close to the physical limits imposed by diffusion, diffraction, and the finite rate of photon detection events. Such an approach has been implemented by Fields and Cohen [84]. The Kalman filter keeps track of both the particle position and the uncertainty with which this position is known.

7. Trapping of single fluorophore with the ABEL trap

A position trace of a trapped Cy5 molecule inside a microfluidic chip in aqueous solution (15 mM HEPES buffer, 30 mM KCl) within the field of view is shown in Figure 5 (c). A representative result of fluorescence intensity time traces with Cy5 in an ABEL trap is shown in Figure 6. We could observe single molecule traces for nearly a second which were followed by single-step photobleaching. This is a tremendous improvement in observation time when compared with the millisecond transit times in a standard confocal microscope. This result clearly establishes the feasibility to confine a molecule within a small region by counteracting its Brownian motion, without canceling this motion completely. Short spikes indicate the occasional entrance of other molecules into the trapping region, or failed trapping attempts. Recent reports by Fields and Cohen indicate an average trapping time of ca. 800 ms prior to photobleaching, with some traces as long as 10 s for Alexa-647 in aqueous buffer media. These achievements are surely going to provoke fresh thoughts and renewed interest in SMS in native environments and under ambient conditions. This will be even more so in the field of biophysics and structural dynamics, where small peptides, proteins, or oligo-nucleotides can be investigated without any disturbances due to surface attachments.

It is worth mentioning that the ABEL trap automatically ensures trapping of a only single objects at a time, because the feedback-generated forces cannot counter-balance

the Brownian motion of more than one molecule at a time. This selectivity is in stark contrast to an optical trap, where any object with sizable polarizibility will be trapped, leading to the build-up or particle agglomerations in the trap over time. Nonetheless, to avoid misleading of the FPGA and the feedback, one generally prefers to work with very dilute solutions, in the concentration range of a few picomolar only. In this concentration range, it is unlikely to find more than one fluorophore within the trapping region at any time.

8. Future perspective and conclusion

Although the ABEL trap is a tremendously significant step forward for long-time and truly single-molecule observation and spectroscopy, it is a very complex and challenging technology. One of its challenging part is the construction and handling of the two-dimensional electrophoretic flow chamber with its exact positioning of the electrodes in respect to the observation region and the nanometer scale confinement along the optical axis. Another challenge is the necessity of a rapid scanning of the laser focus which can currently only be achieved with the necessary speed and accuracy by employing expensive EODs requiring large voltages. Furthermore, the realization of the feedback using FPGAs and sophisticated algorithms is also not a trivial task. However, it became recently possible to make nanofluidic channels which can confine the motion of molecules in two directions on a size-range which is smaller than the typical laser focus of a SMS system [91–94]. Such nanochannels have meanwhile successfully employed for smFRET [95]. We envision that the combination of nanochannel confinement with the idea of the ABEL trap in the third direction will tremendously simplify the application of the technique and will make long-time observations and spectroscopy of single molecules in solution a routine and easy to use tool for many fascinating applications.

Acknowledgments

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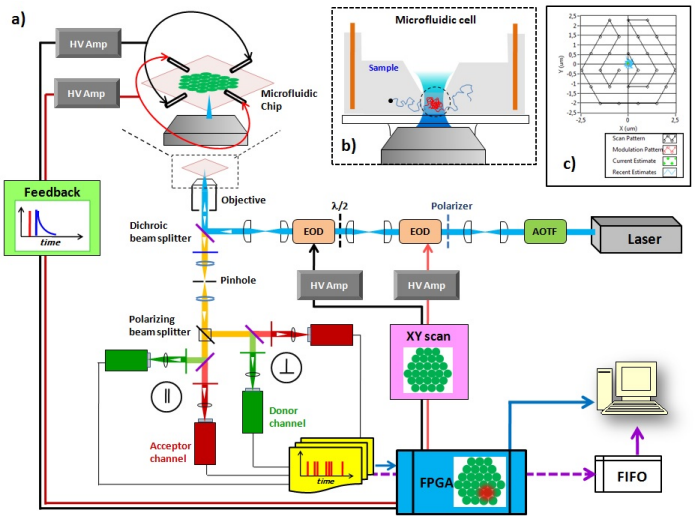

Figure 5. Figure 5: (a) Optical layout of the feedback controlled electro-kinetic trap for single molecule fluorescence spectroscopy. The setup is primarily similar to that of Cohen and Moerner, except the four-channel detection system. Excitation wavelength from a super-continuum laser is selected by an acousto-optical tunable filter (AOTF). The beam is expanded, passed through a linear polarizer (P), and scanned by two electro-optic beam deflectors (EODs). A half-wavelength Fresnel rhomb ($\lambda/2$) rotates the polarization by 900 between the EODs. Relay lenses are used to map the EOD deflections to pure two-dimensional translations in the sample plane. The beam is reflected off a dichroic beam splitter (DBS) and focused on the sample (b). Emitted photons are collected by the objective and filtered through the DBS, polarizing beam splitter (PBS) and an emission filter (F). The emission is focused through a confocal pinhole and subsequently detected by four APDs. Information of detected photons are recorded in FIFO mode and also simultaneously reported to a field-programmable gate array (FPGA), which calculates appropriate feedback voltages which are send to two high–voltage amplifiers (HV Amp) that apply feedback voltages to the sample. The FPGA also sets the deflection voltages applied by the EODs via two additional high-voltage amplifiers. EODs scan excitation light along a set of 27 discrete points with a dwell time of ca. 4 microseconds per point. Spatial information is derived from the location of the laser at the instant each photon is detected. The position of the laser is specified by the FPGA. The scanned images are processed in real-time to extract the x, y-coordinates of a fluorophore of interest and then applies a feedback voltage proportional to the offset between the measured position and a target position. The voltage induces a drift that pushes the particle toward the target position before the arrival of the next x, y-coordinates. (b) The position plot shows trapping of Cy5 in aqueous buffer under feedback controlled electro-kinetic trap.

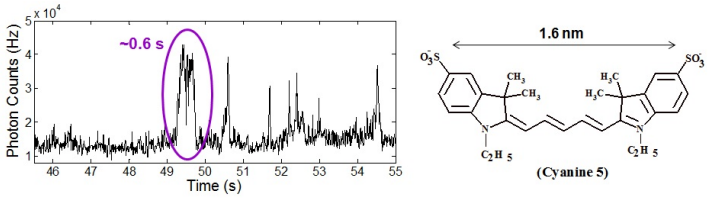

Figure 6. Fluorescence trace (a) of 2 pM Cy5 (b) in 15 mM (pH $\approx$ 7.2) HEPES buffer with 50 mM KCl are shown. To improve the photostability of Cy5, methyl viologen-ascorbic acid and a proportionate mixture of protocatechuate 3,4-dioxygenase, protocatechuic acid and trolox were used as triplet quencher and oxygen scavenger, respectively. The necessary feedback voltage strength is highly dependent on the electrokinetic mobility of the sample and on the geometry of the sample holder, but typically peak voltages of less than 50 V are sufficient.