Applications of nanopipettes in bionanotechnology

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Abstract
At present, technical hurdles remain in probing biochemical processes in living cells and organisms at nanometre spatial resolution, millisecond time resolution and with high specificity and single-molecule sensitivity. Owing to its unique shape, size and electrical properties, the nanopipette has been used to obtain high-resolution topographic images of live cells under physiological conditions, and to create nanoscale features by controlled delivery of biomolecules. In the present paper, I discuss recent progress in the development of a family of new methods for nanosensing and nanomanipulation using nanopipettes.

Introduction
Many important life processes occur in the noisy far-from-equilibrium cellular environment, which is characterized by heterogeneity and stochastic fluctuation. To unravel this complexity, biology requires new physical concepts and methodologies. Currently, there is an increasing demand for the development of nanoscale tools to probe biochemical processes in living cells and organisms with high specificity, nanometre spatial resolution, millisecond time resolution and single-molecule sensitivity.

In the last several decades, micrometre-size glass pipettes have been mainly used for single-ion channel recording in electrophysiology [1] and for microinjection into living cells. When the size of the pipette shrinks from a few micrometres to ∼100 nm or below, new physical phenomena arise, predominantly due to the strong and inhomogeneous electric field in the vicinity of the pipette opening when even a moderate electric potential is applied across the pipette [2,3]. As illustrated in Figure 1(A), the non-linear DEP (dielectrophoretic) force is concentrated at the tip, where the highest field gradient is found due to the conical shape of the pipette. The linear EP (electrophoretic) force opposes and balances this force to trap negatively charged particles at the tip. The EO (electro-osmotic) force is very localized to the surface and dominated by DEP and EP forces. When the polarity of the electric field is reversed, the EP and EO forces invert to deliver molecules out of the nanopipette. Nanopipettes have also been used in SICM (scanning-ion conductance microscopy) [4,5], a non-invasive form of the scanning-probe microscopy family, which is capable of imaging living cells at high spatial resolution under physiological conditions. The controlled delivery [2,6] and trapping [3,7], alone and in combination with SICM, have found many new applications in biomedical sciences [8,9], and the present mini-review aims to highlight recent progress in the development of a family of new methods for nanosensing and nanomanipulation using nanopipettes.

Nanowriting of biomolecules with a double-barrelled nanopipette
Making small features of biomolecules to produce smaller DNA or protein microarrays, or using biological molecules as building blocks to assemble complex structures at defined positions on a surface, are some of the capabilities a glass nanopipette can achieve. Klenerman and co-workers led the way in using the nanopipette as a nanopen to deposit biomolecules on a functionalized glass surface [6,10,11]. Writing with dye-labelled DNA was first demonstrated with a feature size of ∼800 nm [6], followed by the creation of complex structures using the self-recognition properties of biological molecules [10]. By using a double-barrelled nanopipette, it is possible to independently deposit different species sequentially [11]. As shown in Figure 1(B), the double-barrelled nanopipette is operated in air with the water meniscus formed between the pipette and the surface. The voltage between the two barrels produces the distance-feedback control as well as the control of molecule delivery. Since the lateral diffusion of the delivered molecules is restricted within the water meniscus, a smaller feature size can be obtained compared with a single-barrelled pipette operating in solution. The technique could be extended to multi-barrelled pipettes with up to seven barrels so that a larger number of species could be deposited with the same pipette, and thereby greatly increasing the complexity of patterns generated. The application of double-barrelled nanopipettes in nanoscale biological assays has been demonstrated by producing arrayed water droplets with volumes as small as a few attolitres under an organic layer [12]. Individual droplets can be addressed, and controlled amounts of either additional volume or reagents can be added from one of the barrels of the pipette [12]. The method could be used for miniaturized cell-free protein expression in an arrayed format.
Figure 1 | Applications of nanopipettes in dielectrophoretic trapping, nanowriting, nanosensing, and single-molecule and single-cell manipulation

(A) Schematic diagram of the forces and flow inside the nanopipette tip that enable manipulation of negatively charged particles (green). (a) Non-linear DEP force (blue) is concentrated at the tip, whereas linear EP force (red) opposes and balances this force to trap particles at the tip. The EO force (yellow) is very localized to the surface and negligible. (b) By reversing the polarity, the EP (and EO) forces invert to deliver particles out of the nanopipette. Figure courtesy of Dr Joseph Piper. (B) Principle of biomolecule deposition using a double-barreled nanopipette. The two barrels of the pipette are filled with different fluorophore-labelled antibodies for deposition on to a functionalized glass surface. The voltage between the two barrels produces the distance-feedback control current and also controls the molecular delivery. Adapted from Rodolfa, K.T., Bruckbauer, A., Zhou, D.J., Korchev, Y.E. and Klenerman, D., ‘Two-component graded deposition of biomolecules with a double-barreled nanopipette’, Angewandte Chemie International Edition, 2005, vol. 44, pp. 6854–6859. Copyright Wiley-VCH GmbH & Co. KGaA. Adapted with permission. (C) Upper panel: schematic diagram of the renewable nanosensor. The reporter dye molecules are trapped in the nanopipette tip. Analyte ions diffuse into the tip and bind to the reporter molecules changing their fluorescence. This fluorescence is then collected by the confocal optics. Lower panel: fluorescence mapping of the sodium nanosource by the nanopipette sensor. Adapted with permission from [21]. Copyright 2006 American Chemical Society. (D) Left-hand panel: localized melting of individual dsDNA molecules as 1 M HCl is dosed from a nanopipette brought close to the surface. The single-molecule dissociation triggered is detected by the disappearance of FRET signal. Right-hand panel: experimental setup for manipulating the rotation of a single E. coli sodium-driven chimaeric flagellar motor. The nanopipette doses sodium with an applied voltage and causes the flagellum to rotate faster. Detection is via a camera that images the bead illuminated by the microscope lamp. Adapted with permission from [22]. Copyright 2008 American Chemical Society.

**Trapping of biomolecules with a nanopipette**

We have found that DNA can be trapped inside glass nanopipettes near the tip, which results in a concentration enhancement of a factor over 1000 [3]. The trapping can be attributed to the interplay between EP, DEP and EO forces. A strong trapping effect was observed during the negative half-cycle for all DNA samples. This effect was more...
Manipulating conformation of donor/acceptor-labelled DNA to function as a molecular switch

The use of nucleic acids as molecular switches or nanomachines is currently of great interest. Most DNA conformational switches presented to date are slow to switch over because either bimolecular hybridization or a change of buffer is required. The feasibility of developing a DNA switch that overcomes these problems was investigated by taking advantage of the strong local electric field near the tip of the pipette to alter the DNA–dye interaction which renders the switching process rapid (<100 ms) and reversible without by-products [15]. The DNA switch is based on a 40-bp dsDNA labelled with a donor fluorophore, Rhodamine Green, and an acceptor fluorophore, Alexa Fluor® 647 [a Cy5 (indodicarbocyanine) derivative]. The fluorophores were attached to the same end of the duplex. It was found that DNA molecules can be reversibly switched between a donor-emitting green state and an acceptor-emitting red state upon the application of an electric field inside the glass nanopipette. The electric field appears to alter the conformation of the acceptor dye only, which results in a significant change in its fluorescence quantum yield, presumably owing to interactions with the DNA strand. The single-molecule DNA switch was characterized further in free solution at the single-molecule level to elucidate the fluorescence-switching mechanism [16]. It was found that the acceptor Alexa Fluor® 647, which is in an initial high fluorescence trans state, undergoes a photoisomerization reaction, resulting in two additional states: a non-fluorescent triplet state, a singlet state, and a singlet cis state that blocks the FRET (fluorescence resonance energy transfer) pathway and gives rise to donor-only fluorescence. The formation of these states is faster than the diffusion time of the DNA molecule, so that all three states are approximately equally populated under experimental conditions. The acceptor dye can stick to the DNA in all these states, with the rate of unsticking determining the rate of isomerization into the other states. Measurement of the rate of change of the FRET signal therefore provides information about the fluorophore–DNA intramolecular dynamics. This finding indicates that the conformations of other biological molecules as well as voltage-gated ion channels may be switched by the application of an electric field. It is worth noting that the photoisomerization mechanism of the cyanine dye has been recently exploited in super-resolution fluorescence microscopy [17].

Nanomixer for single-molecule kinetics measurement

A new methodology has been developed for studying reaction kinetics at the single-molecule level by incorporating a glass nanopipette into a confocal optical system to form a quasi-continuous-flow nanomixer [18]. It was found that the mixing of biomolecules with the bulk solution is predominately by diffusion, therefore the relation between the time at which there is maximal probability to observe the molecules and the distance from the pipette opening can be derived based on Einstein’s three-dimensional diffusion equation. The nanomixer was demonstrated first by measuring the dissociation of DNA duplex and then applied to the study of intramolecular quadruplex unfolding by directly monitoring the subpopulations of the different conformations of a quadruplex during unfolding using single-molecule FRET, which provided kinetic information complementary to previous work. This nanomixer offers a slightly better time resolution and approximately the same timescale as Eaton’s microfluidic system [19]. This new methodology has the added advantages of a reduced background, as measurements are made in free solution, and, more importantly, simple device fabrication, which should make the method widely applicable. It can be applied to studies of important biological processes, such as protein folding, under non-equilibrium conditions in an experimentally simple manner. This has been demonstrated very recently by a study on Citrine, a variant of green fluorescent protein [20]. The nanomixer was used
to detect an intermediate on the unfolding/folding pathway directly and the existence of parallel unfolding pathways was also revealed.

**Renewable nanosensor for metal ions and pH sensing**

As illustrated in Figure 1(C), a renewable nanosensor has been designed based on the principle of DEP trapping, where a reporter dye molecule is trapped and concentrated in solution at the nanopipette tip [21]. The reporter is excited by focusing a laser beam using far-field optics at the 100-nm inner diameter pipette tip, producing a local nanosensor where the fluorescence is then dependent on analyte concentration in the bath. This has the flexibility of trapping essentially any reporter dye molecule at the tip in solution without copolymerization or extensive preparation. SNARF-1-dextran, a negatively charged ratiometric pH-sensitive fluorophore, and negatively charged CoroNa Green dye were used to demonstrate the concept of the nanopipette as a pH and sodium sensor respectively. The key to this method which distinguishes it from others is that the dye at the tip is renewed from well inside the nanopipette so that the reporter dye is replenished, enabling the sensor to be used for a prolonged period and thus overcoming photobleaching problems. The nanosensor has a time resolution of ∼2 ms and a spatial resolution of ∼600 nm, which can be improved further by reducing the probe–sample distance. This technique makes it possible to map the spatial variation of the analyte concentration distribution arising as a result of local biochemical reactions or processes, therefore it can be applied to nanoscale mapping of analytes over living cells.

**Controllable local chemical change as a tool for single-molecule and single-cell manipulation**

A versatile method that allows local and repeatable delivery (or depletion) of any water-soluble reagent from a nanopipette in ionic solution to make localized controlled changes in reagent concentration at a surface has been presented recently [22]. Na⁺ or OH⁻ ions were dosed from the pipette using pulsed-voltage-driven delivery. TIRF (total internal reflection fluorescence) from CoroNa Green dye in the bath for Na⁺ ions, or fluorescein in the bath for pH, quantified the resulting changes in local surface concentration. These changes had a time response as short as 10 ms and a radius of 1–30 μm, depending on the diameter of the pipette used, the applied voltage and the pipette–surface separation. This opens a new route for single-molecule and single-cell manipulation. Two proof-of-concept experiments were performed as shown in Figure 1(D). Individual duplex DNA molecules were dissociated by dosing acid locally from the pipette to the immobilized molecules. The melting process was monitored by single-molecule FRET. The rotation of a sodium-sensitive flagellar motor in a single *Escherichia coli* chimaera was also shown to be controllable on the timescale of 1 s by dosing sodium and monitoring the rotation of a 1-μm-diameter bead fixed to the flagellum. This new approach is capable of studying protein folding and protein–protein interaction at the single-molecule level by targeted delivery of chemical denaturants such as guanidinium chloride and functional biological molecules. It may also find applications in the mechanistic study of chemotaxis by creating nanoscale chemical gradients. Furthermore, since the pipette can be scanned over a surface without contact using ion-conductance distance feedback, it becomes feasible to map the chemical reactivity of the surface of living cells.

**Summary**

Nanopipettes are versatile tools for sensitive detection and manipulation on the nanoscale and they are finding increasing uses in many areas of biomedical sciences. The present mini-review provides a glimpse of what this simple device can offer to bionanotechnology. New applications are continuing to emerge [23–24]. One area to look at is the integration of nanopipette manipulation with single-molecule fluorescence imaging [25]. A nanopipette could be used to deliver individual biomolecules through the tip of the pipette, which are then landed on to a specific site of the cell surface and allowed to bind to cell receptors, thus triggering a cascade of cell signalling events which could be followed by multicolour single-molecule tracking [26]. This will allow the study of complex signalling networks with nanometre spatial resolution and millisecond temporal resolution at the single-molecule level.

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**References**


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