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Single-molecule trapping and measurement in solution

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Trapping of a single molecule in the fluid phase was realized decades following developments in the gas-phase, because in some ways the solution phase posed a greater challenge. The key issues have since been addressed by several different means; techniques to confine nanometer scale entities in solution now abound and are gaining traction in a variety of single molecule studies. Available methods range from pure physical entrapment of a molecule on the one hand to electrokinetic and optical techniques, and approaches that exploit thermodynamic principles on the other. Some trapping techniques have also opened up new avenues to highly precise, accurate measurements of molecular physical properties in solution.

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Since the advent of single molecule optical detection in the condensed phase [1,2], imaging and detection of emitters using visible light has become a methodological cornerstone in the quest to measure the properties and understand the dynamics of molecular scale matter in solution. The short timescales associated with free diffusion in solution however generally render molecules in motion invisible above the noise floor of the detector and/or measurement background. Experiments on optically emitting molecules have therefore generally relied on physical attachment of the entity of interest to a surface in order to facilitate long term observation, with immobilization and related methods such as encapsulation, tethering, and confinement using nanostructured surfaces serving as the mainstay of biomolecular imaging experiments [3–5].

Following the cue from the century-old effort in the gas phase, external fields may be used to quell thermal motion and to spatially confine a molecule in solution. However in general, the trade-off between thermal energy and say, the free energy of the field-induced dipole in a macromolecule, turns out highly unfavorable in room temperature solution [6]. In other words, potential wells created by approaches capable of spatially trapping cold atoms at one extreme and micron-scale particles in a warm fluid on the other, are far too shallow in the intermediate regime of the small, weakly polarizable, and warm, invariably encountered when dealing with biomolecular matter in room temperature solution. So novel trapping approaches have had to be developed in order to address the unique challenges posed by the solution phase.

Physical confinement approaches for long term observation of single molecules

Beginning with an early demonstration of elastomeric microfluidics in single molecule experiments, approaches based on the soft elastomer polydimethylsiloxane (PDMS) have continued to provide a robust route to confining single molecules in solution [7,8]. Initial single molecule enzymology experiments in passive femto-liter reaction chambers have given way to approaches that further exploit the soft and reversibly deformable nature of PDMS [9,10]. ‘Flexible confinement’ has proven a highly useful and novel development in that it permits rapid alternation between bulk-like and confined states, supporting mixing, exchange, and rapid transport of material into and out of the vicinity of the individual molecule of interest, as demonstrated in a recent comprehensive study at the molecular scale [10]. Along similar lines, convex-lens induced confinement and nanotemplating is a simple, versatile approach that uses a lens in near contact with a nanostructured substrate to generate molecular confinement [11–13]. Banterle and Lemke provide an exhaustive review of recent developments in confinement-based approaches for single molecule experiments [14] (Figure 1).

An overview of techniques offering long term observation of molecules in solution would not be complete without reference to the refinement in 3d tracking microscopy that now permits direct observation via particle tracking of a single molecule freely diffusing in solution [15]. Although the approach offers lower molecular throughput than the nanostructure-based techniques, it does directly address and solve the key challenge of long term observation without recourse to any confinement or trapping principle.

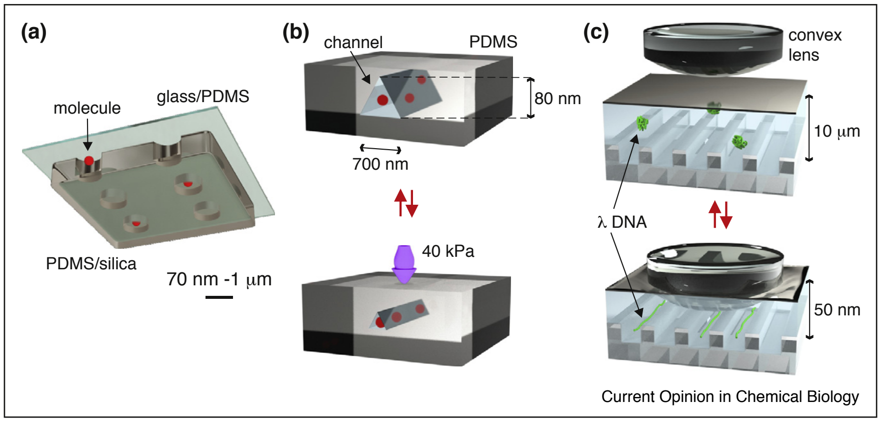
Trapping single molecules in solution

Direct spatial control of single molecules diffusing in solution remained elusive until the invention of the anti-Brownian electrophoretic trap (ABEL trap) a little over a decade ago [16]. The approach furnished single molecule fluorescence-based feedback-control of the position of a molecule in solution using applied electric fields and has been realized in one [17,18•] and two dimensions [16]. Subsequently, other field-based approaches utilizing deterministic AC electrical fields [19,20••], near field optical effects [21,22,23••], and temperature gradients [24•], have demonstrated the ability to directly confine nanoscale matter and large macromolecules in solution. Passive trapping approaches relying on equilibrium thermodynamics rather than field-driven or gradient-driven transport have been further developed, now not only addressing the need for long term observation but importantly enabling new approaches to high precision measurement of molecular properties in solution [25,26••]. Below we briefly discuss state-of-the-art trap-based approaches to molecular control in free solution, their unique capabilities and limitations, and the prospects held out by the most recent developments in this burgeoning field of research (Table 1).

Spatial control based on electrical mobility

Most biological macromolecules carry some net electrical charge, either positive or negative. Motion of a charged molecule in a uniform electrical field in solution arises both from the response of its electrical charge to the local field charge (electrophoresis) as well as from field-driven flows due to possible charge non-neutrality in the surrounding fluid (electro-osmosis). While Earnshaw's theorem forbids the realization of a stable spatial trap for an electrical charge in a static field, time-dependent electric fields — both stochastic and deterministic — can confine charged objects in free space and solution [16,19]. The ABEL trap for example, exploits an electrical feedback-based mechanism requiring continuous monitoring of single molecule fluorescence [27–29] (Figure 2a). An analogous approach using a similar planar electrode configuration is the Paul trap in the fluid phase, with the important distinction that deterministic electrical fields are used in order to attain time-averaged stable confinement, and particle observation is not required [19]. The challenge in scaling down the Paul trap to the molecular level appears to be the high frequencies involved which have concomitant heating effects. Nonetheless it would be a welcome development with important implications for measurement if this trapping principle which has been so successfully deployed in the gas phase were able to deliver promising results for molecular scale matter in solution. Finally, replacing the DC field used in molecular separations with an alternating field applied to an effectively one dimensional nanocapillary, Lesoine *et al.* [18•] have demonstrated a straightforward route to trapping molecules in solution (Figure 2b). The key feature of this approach is that it permits trapping of single molecules without the need for continuous observation, which considerably reduces the photobleaching rate and increases the overall trapping time. Furthermore, the one-dimensional scheme supports parallelization, and may also facilitate feedback-free operation, similar to the aqueous Paul Trap.

Figure 1



Confinement approaches for long term observation of single nanoparticles and molecules. **(a)** Attoliter to femtoliter silica [8] or PDMS [7] chambers. Particles are confined in closed nanoscopic chambers created between a glass surface and PDMS layer, facilitating long term parallel observation of single molecules. **(b)** Size-adjustable elastomeric micro-channels. Compressing a channel with micrometer scale cross-sectional dimensions reduces its dimensions to the order of the point spread function of the optical excitation. This permits visualization of molecules diffusing in one dimension in a wide field fluorescence microscope. Removal of the applied force restores the channel to its original form, thus enabling mixing and transport of molecules and solutes [9]. **(c)** Convex lens induced confinement and nanotemplating utilizes tunable contact between a convex lens and a substrate carrying nanoscale topography to generate reversible spatial confinement of molecules [13].

Table 1

The table presents a comparison of trap-based methods for single molecule confinement and measurement in solution. Parameters noted are: longest reported trapping time for a single molecule in a single trap; the smallest reported trapped molecule and its approximate mass; number of individual molecules that can be trapped in parallel and observed in the same field of view; prospects for simultaneous confinement of more than one molecule in a single trap. Finally we list a key feature of each technique that currently poses a challenge but whose resolution would present a signification future advance

Trapping mechanism		Time	Size	Number	Multimolecular trapping	Future developments
Feedback-based electrokinetic trapping	ABEL	~5 min (eGFP) [49]	Organic fluorophores (1 kDa) [41]	1	No	Scattering detection [50]
	Recycling	>15 s (dsDNA) [18•]	13 bp dsDNA (8.5 kDa) [18•]	1	No	Scattering detection, feedback-free operation
Nano-aperture optical trapping		~3 min (Streptavidin) [23••]	20 b ssDNA (6.5 kDa) [34]	600 [23••]	Yes	Preserving molecular structural integrity
Thermophoretic trapping		>8 min (λ-DNA) [24•]	λ-DNA (~30 M Da) [24•]	1	Yes	Trapping molecules <1 MDa
Geometry-induced trapping	Electrostatic	~30 min (disordered protein, Stm-I) [26••]	Organic fluorophores (1 kDa) [40•]	>4000	Yes	Operation in ionic strengths >10 mM
	Dielectrophoretic	Seconds (immunoglobulin G antibody) [20••]	IgG antibody (~144 kDa) [20••]	~1	Yes	Trapping small molecules (<140 kDa)

Beyond transport arising from net electrical charge, electrical mobility can also arise from a dipolar response in a field gradient, even if the molecule has no intrinsic electrical dipole. Although dielectrophoretic (DEP) forces have been used for concentration and local spatial enrichment of molecules [30] in high field regions, stable spatial trapping of an individual molecule over longer periods appeared harder to achieve. A recent demonstration of DEP concentration in conjunction with nanopore-based conductance sensing highlights some of the issues encountered in attempts to realise DEP trapping in free solution [31]. However reduction in system dimensions to an effectively one dimensional nanochannel elegantly circumvents these challenges and has recently enabled confinement of single macromolecules in regions of low electric field [20••], as discussed in the final section.

Optical control of matter beyond the focused beam trap

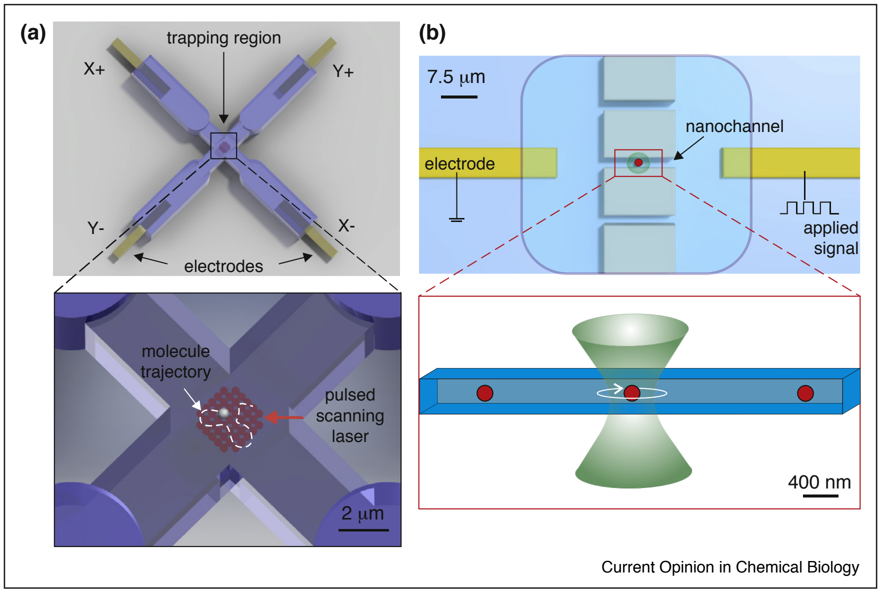
Trapping particles in a focused optical beam in free solution has a rich and long-standing tradition in experimental biophysics [32]. However the depth of the minimum in dipole energy in a free-space optical focus scales with the third power of object size, rendering the optical gradient force alone infeasible at the molecular scale in room temperature solution [6]. Recently, novel optical techniques have been developed that substantially enhance the gradient force, or harness different physical principles in addition, thereby not only permitting trapping of single biomolecules in solution but also enabling new forms of optical measurement on molecules using low optical powers (Figure 3).

The self-induced back action (SIBA) optical trap is a new concept in optical trapping at the nanometer scale [21,22,23••]. The technique exploits conservation of momentum in the transmission of light through a nanoaperture in a metal film. Briefly, the motion of a dielectric object away from the nanoaperture results in a reduction in transmission and hence photon momentum through the aperture. This loss in momentum in turn creates a restoring force on the particle toward the aperture. The method also supports measurements of changes in mass or conformation of a molecule by monitoring the modulation of transmitted light through the aperture.

In contrast to 'nanoparticle-based' plasmonic traps such as bowtie antennae, where resonant excitation produces high local gradients in the electromagnetic field, aperture-based traps do not require a high quality resonance. The nanoaperture approach also benefits from efficient dissipation of thermal energy through the metal film preventing the buildup of high local temperatures [33]. The strong 'back-action force' supports very long molecular residence times in the nanoaperture on the order of several minutes, or even hours [34]. Furthermore the high temporal resolution in monitoring transmission through the aperture could enable high resolution measurement of attributes of molecular states such as 3D conformation [33].

A novel measurement method developed using the SIBA experiment takes advantage of the inherent acoustic vibrational modes of the molecule of interest [35••,36]. Detuning a second laser so that the beat frequency matches that of an acoustic mode of the molecule, a resonant vibrational excitation occurs which causes local

Figure 2



Electrokinetic approaches to single molecule trapping. **(a)** The Anti-Brownian Electrokinetic trap relies on a scanning laser beam and fluorescent feedback from a diffusing single molecule to electrically cancel the effect of Brownian motion and restore the molecule to the center of the trap [16,29]. **(b)** The 'recycling trap' reduces the problem to one dimension using a nanochannel to confine the molecule [18•].

heating. Thermal motion of the molecule is altered which manifests in the fluctuation amplitude of light transmitted through the aperture. The molecule's low frequency vibrational spectrum can be recorded by monitoring thermal motion over a range of beat frequencies. Although the mechanism remains to be fully understood, the observed resonance frequencies match the Raman spectrum of the molecule, opening up new avenues for spectroscopy at the single molecule level. Differences in length and base sequence in single stranded DNA have been detected by this approach with prospects for detection of single base differences [36]. Protein interactions with DNA [37] or smaller molecules [38] have also been probed using this technique.

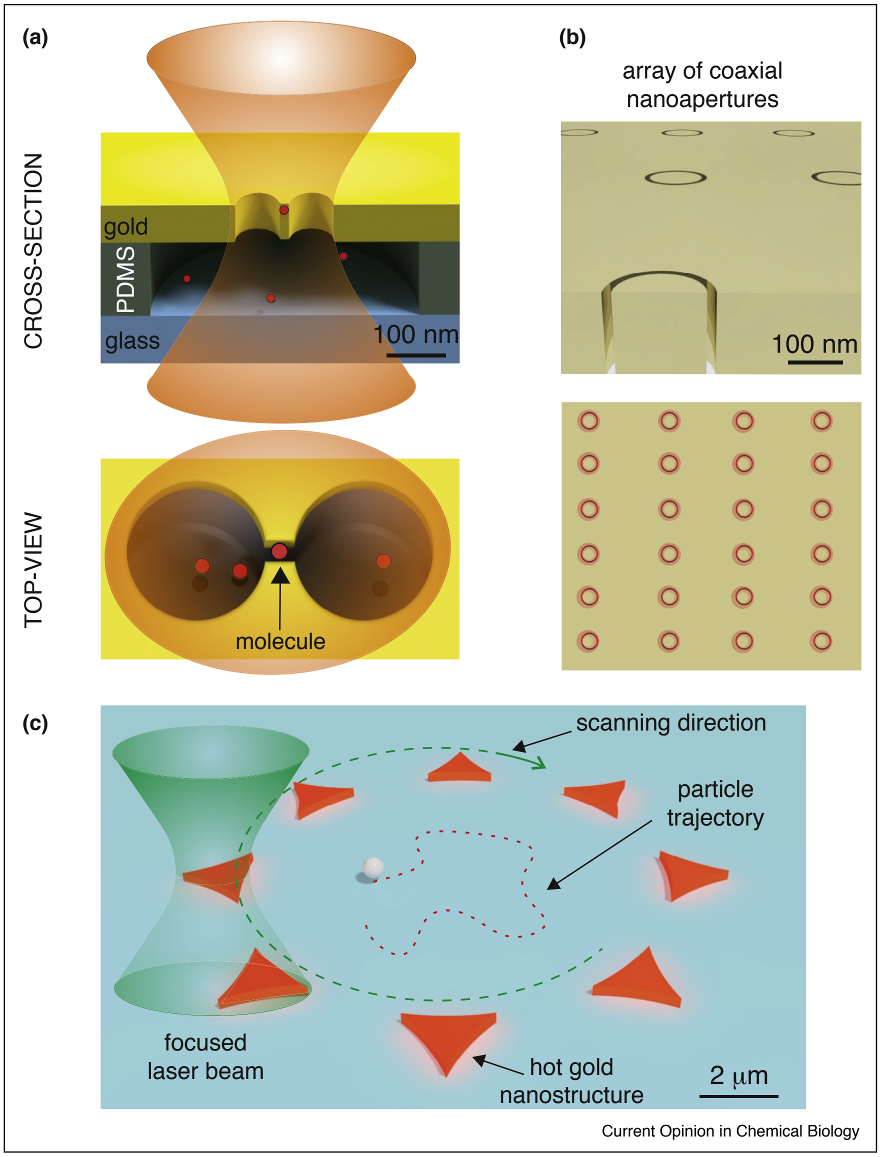
However, since proteins are essentially deformable dielectric objects, early realizations of molecular trapping in double nanohole apertures reported unfolding of trapped proteins [22]. It is likely that this aspect may be addressed through the use of different aperture geometries such as the coaxial nanoaperture [23••] (Figure 3b), in which case this new optical approach could turn into the method of choice for a wide variety of single molecule studies.

Thermophoretic trapping

The Soret effect refers to the motion of molecules and ions in solution in response to a spatial temperature gradient. Molecules travel either up or down a temperature gradient depending on the value of the Soret coefficient, which is about $1\,\mathrm{K}^{-1}$ for a large DNA molecule [39]. Although the microscopic details of the process are not yet fully understood, particle motion is attributed to a temperature-induced spatial entropic gradient, entailing contributions from both the screening ions in solution as well interfacial water [39]. A spatial gradient in temperature therefore drives a molecule in solution to a location that minimizes the free energy of the system.

Braun *et al.* recently demonstrated the ability to use optically induced heating of a structured gold film in order to create the necessary spatial temperature profile in solution [24•] (Figure 3c). Large thermophoretically induced potential well depths, $U = S_T\,\Delta T > 10\ kT$ where k is Boltzmann's constant and T is the absolute ambient temperature, can be attained for certain macromolecular species. The requirement of a temperature difference of $\Delta T \sim 10\,\mathrm{K}$ to stably confine λ–DNA which has a Soret coefficient, $S_T \sim 1\,\mathrm{K}^{-1}$ however suggests that current experiments may have already attained the approximate size-limit of the approach. For example, S_T scaling as $\sqrt{L}$ for double-stranded DNA would imply a lower bound of 1000 bp for thermophoretic trapping of single DNA molecules [24•]. Nonetheless it remains to be seen if variations on the basic theme behind this conceptually novel approach — perhaps involving different ionic species in solution, or combination with other effects — may support operation in the regime of molecular dimensions approaching 1–10 nm.

Figure 3



Trapping based on the optical near-field and thermophoretic trapping. **(a)** Illumination of double nanohole apertures in a metal film confines single molecules in the cusp region between the nanoholes (≥5 mW in the optical focus) [22,23••]. **(b)** High density arrays of coaxial nanoaperture traps can confine molecules using low laser powers, <5 mW [23••]. **(c)** Plasmonic nanostructures heated by a scanning laser beam generate a radial temperature gradient in solution that traps single nano-objects via thermophoresis [51].

Geometry-induced trapping in nanoscale systems

Electrostatic and entropic trapping offer 'field-free' confinement

We recently demonstrated the use of an equilibrium thermodynamic principle that exploited geometry-induced local spatial minimization of free energy to trap single charged molecules in solution [26••]. Molecules confined in solution in a thin gap between like-charged parallel plates experience repulsive interaction energies everywhere in the slit except at locations where one of the slit walls carries a nanoscale surface indentation. At these locations, the slit surfaces are farther away and the repulsive interaction energy between the plates and object is smaller; in fact the free energy of interaction is set to go to zero by design, creating a stable thermodynamic trap for the object of interest. The approach not only permits trapping of single molecules without the application of any external fields, but it also provides sub-diffraction limited spatial confinement and highly parallel trapping of single entities (Figure 4a). Importantly, trapped molecules spend most of their time in a 'field-free' region where the electrical potential is zero, and thermal equilibrium guarantees that the solution conditions are identical to those in the bulk (Figure 4a). This feature of geometry-induced trapping — using pure electrostatics, and possibly to some extent in the dielectrophoretic version as well (discussed next) — sets it apart from all other molecular trapping approaches where the trap is always a region of significant field.

We have shown that measuring the average residence time of a single molecule in a trap permits molecular electrical properties such as net charge, spatial charge distribution and interior dielectric constant to be measured with high precision, or inferred given additional information such as chemical composition [26••]. Trapped molecules migrating or 'hopping' through a lattice of traps can be followed individually in real time over a period of minutes or hours in order to extract information on slow dynamic molecular processes. Although operation of the electrostatic fluidic trap in its current form is confined to salt concentrations in the low mM range, improvements on this front may be expected in the future through the use of surface functionalization of the slit walls. Importantly, we demonstrated in a recent study that 'entropic enhancement' of geometry-induced trapping free energies could deliver all the advantages of electrostatic trapping while supporting operation in higher ionic strength solution, say 10 mM or higher [40•].

Dielectrophoretic trapping

The concept of geometry-induced modulation of an electrical interaction energy can be extended beyond the monopole electrostatic picture described above to the minimization of dipole energy in a spatially varying electric field. Electrodes in trough regions of a geometrically modulated nanochannel can be used to generate the required electric field gradients (Figure 4b). The frequency-dependent polarization response of an object in an electric field results in the formation of an effective molecular dipole that is either repelled or attracted to regions of high field. At very high field frequencies (~1 GHz) the system response is dominated by permittivities rather than conductivities and low dielectric nanoparticles in water seek field minima. At intermediate frequencies the qualitative response (positive or negative DEP) results from a balance between effective particle and medium conductivities. Using applied fields of about 1 V/μm at frequencies of 10 MHz in nanochannels with constrictions of cross sectional dimension ~70 nm permits confinement of 144 kDa Immunoglobulin G molecules (diameter ca. 10 nm) in solutions of 16 mM ionic strength [20••]. Although the unfavourable third power scaling of dipole energy with molecular size may be offset by scaling down the dimensions of the constriction in this approach [20••], all things considered well depths larger than ~1 kT, where k is Boltzmann's constant, will be challenging to attain for smaller molecules (Figure 4b).

It is worth noting that of the current molecular trapping techniques, the ABEL trap and electrostatic fluidic trap are the only approaches that have demonstrated the ability to confine a single small molecule such as an organic fluorophore in solution [40•,41]. Furthermore although dissociative charging of an ionizable group is not energetically favoured in an apolar medium and electrostatic interactions are expected to be weak compared to water, the long screening length attainable permits the electrostatic fluidic trap to work surprisingly well in organic solvents [42]. This could open up possibilities for single molecule experiments in, for example, polymer chemistry [43].

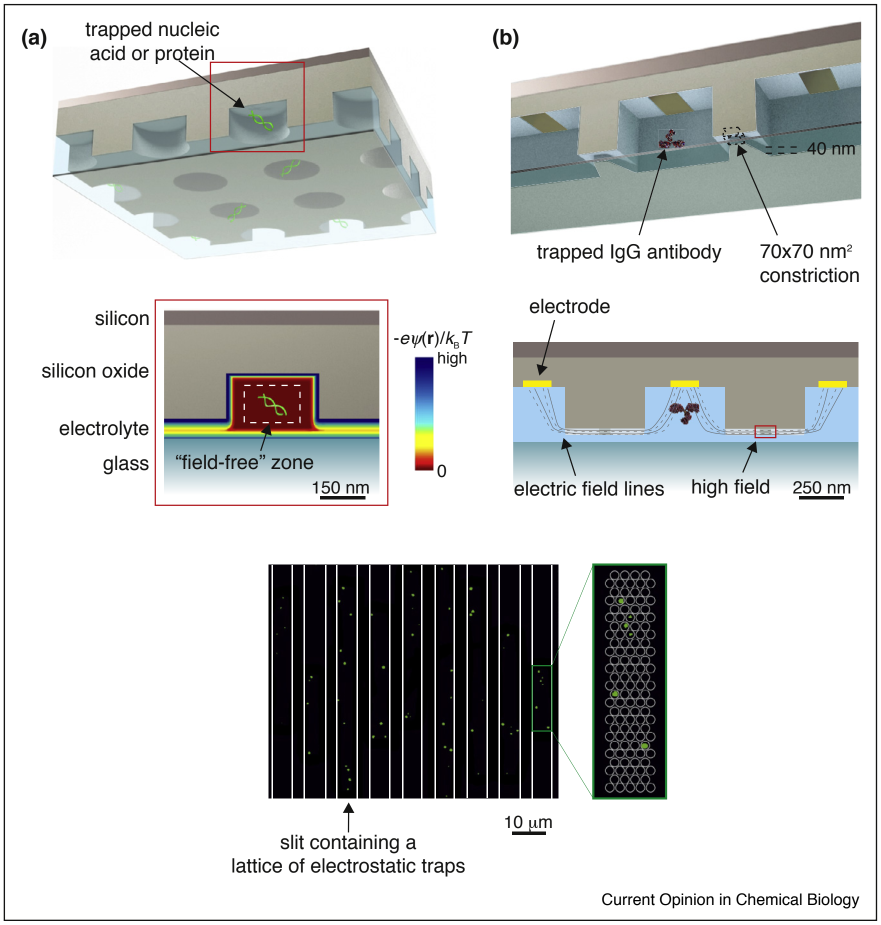
To summarize, realization of a technique that meets every single requirement of non-destructive or non-invasive trapping or confinement of a small molecule that offers long term (continuous observation time ~ seconds or minutes), strong spatial confinement (diffraction limited), in room temperature physiological solution conditions is yet to fully materialize; although most experiments will not require all these criteria to be met. The interesting question is now turning into what single molecule trapping can offer beyond long-term observation in the solution phase.

New science using single molecule traps

The effort to trap single molecules in solution has spawned new measurement techniques in molecular spectroscopy as well as approaches to addressing fundamental questions in molecular transport and the nature of the molecular interface in the fluid phase. For example, the nanoaperture optical trapping measurements have furnished an unanticipated novel way of measuring acoustic vibrational modes of single molecules [33,36]. Traps exploiting transport and thermodynamic principles have opened up the unique possibility of making high precision measurements of transport and physical properties of molecular species, including mixtures, at the single molecule level. Examples of such properties are electrical mobility [44••], translational [44••,45] and rotational diffusion coefficients [29], oligomeric state [44••], conformation [28], photophysical properties of pigment proteins [27,46], binding constants [38,44••], net electrical charge, non-uniform charge distribution, and dielectric constant of the globular interior of, for example, a folded protein [26••]. Furthermore, many of these approaches may enable careful examination of intermolecular interactions by controlling the precise number of molecules in a single trap.

Single molecules serve as highly well defined probes of interaction energies in solution. Relating accurate experimental measurements to the appropriate theory will likely foster a deeper understanding of the underlying phenomenology and the discovery of new effects. For instance, the ability to probe the transport and interactions of a single well-defined entity such as short dsDNA fragment in

Figure 4



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Geometry-induced electrical trapping. **(a)** The electrostatic fluidic trap [25,26••,40•]. Schematic representation of the fluidic nanostructure used to generate a lattice of electrostatic traps in solution (upper panel). Spatial electrostatic potential distribution in a single trapping nanostructure. The dashed outline indicates the 'field-free' region in the trap where the electrical potential is zero and solution conditions (e.g. pH and salt concentration) are identical to those in the bulk. The energy scale shown is that of a negative point charge (middle panel). Representative wide field fluorescence microscopy image of single ATTO532 labelled 60bp DNA fragments in a lattice of electrostatic fluidic traps. The image presents a very low density of molecules (ca. 50 in the field of view), but high densities are now routine (Table 1). Locations of traps are indicated by circles and individual nanoslits bearing high-density lattices of traps are demarcated by vertical lines (lowest panel). **(b)** Dielectrophoretic trapping in a nanochannel [20••]. AC fields applied at electrodes in trough regions of a corrugated nanochannel create zones of high electric field at channel constrictions. At high frequencies energy barriers in the high-field zones enable confinement of macromolecules in the troughs. The approach has been used to demonstrate confinement and field driven sorting of large macromolecules. Depicted molecules are not to scale.

solution in a size-dependent manner may shed light on poorly understood phenomena such as the Soret effect and interfacial solvation. Precise measurements of electrical interaction free energies in solution could enhance our understanding of ion-specific effects in solution phase electrostatics, and possibly shed light on the long-standing "like-charge attraction" problem [47,48]. Measurements of electrical mobility at the single molecule level may enable more accurate comparisons and benchmarking against theoretical predictions, not possible previously due to poorer measurement accuracy inherent in ensemble-averaging experimental approaches. Many open questions in soft-matter and liquid state science, specifically in colloid physics, molecular biophysics, interfacial phenomena and electrohydrodynamics, may be addressed by the kind of high precision molecular level measurements enabled by single molecule trapping techniques. These advances may well be accompanied by the development of new ultrasensitive biomolecular detection technologies.

Conflict of interest statement

Nothing declared.

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