"Microfluidics for Ultrasmall-Volume Biological Analysis"

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Biochemical analysis on a very small scale is beneficial—anyone that has given any thought to the subject could define a variety of reasons for this: small samples lead to minimally invasive clinical/diagnostic procedures, multiple analytes can be measured from single traditional samples, most very small scale procedures lead to faster turn around and present the possibility of massive parallel analysis, and certainly less waste is generated. A less obvious aspect of small volume chemical analysis on a fast time scale is that it will soon be possible to monitor biology on the same time scale and volume in which the intricate mechanisms of biochemistry occur in living systems. In fact, for microelectrodes, microdialysis and near-field fiber optic probes this capability could be argued as a reality, albeit for a small subset of the interesting active biomolecules. This basic research idea of intimately monitoring the chemical activity of living systems has essentially driven the field of ultrasmall volume bioanalysis; although it is unclear if the lose collection of techniques and methods that are utilized for these analyses even constitutes a defined field.

Origins

The idea of ultrasmall sample volume brings with it a number of new problems and opportunities. The relatively new term used to describe controlling ultrasmall fluid volume, microfluidics, defines a class of manipulations where commonplace macro scale notions of transport no longer dominate the movement of fluids and molecules within a fluid. Before embarking on a cursory understanding of developments in the field, it is important to define of the parameters of microfluidics. For example, the flow stream and sample volumes: typical volume flow rates range from a few nanoliter/sec to picoliter/sec and sample volumes from nanoliter to femtoliter. Just to emphasize how small a

femtoliter (10^{-15} L) is, it is instructive to look at it common notation: some 1/1,000,000,000,000,000 of a liter. On these small scales gravity and convection are no longer the dominant forces; surface tension, intermolecular and surface interactions, and diffusion typically dominate motions and interactions. Microfluidics and ultrasmall volume bioanalysis can trace its roots along several paths (Table 1, Figure 1): From microscopy, histology, and immunohistology; from nano and micro chemical probes such as microelectrodes, near field fiber optic probes, and ion selective electrodes; from microdialysis sampling, capillary electrophoresis and flow cytometry; from advances in detection technology in spectroscopy, mass spectrometry and molecular recognition; and from recent developments in microfluidics on microchip devices.

Origins of Microfluidics	
• Microscopy	• Microdialysis
• Histology	• Capillary Electrophoresis
• Immunohistology	• Detection Technology
• Probes (Nano and micro)	• Molecular Recognition
• Flow Cytometry	• Fabrication Processes

Table 1

Microfluidics grew out of a number of different fields, each with its own contribution to the state of the art in ultrasmall volume biological analysis.

Microscopy

Building on this theme of multiple origins, it is instructive to briefly review the various lines of development that have brought about the present technology. Certainly, it is easiest to start with light microscopy of excised biological samples as the origin of ultrasmall volume samples, where the visualized structures were correlated with healthy and disease states, and eventually with the underlying biochemical processes. Recently, the visual images have been extended to native fluorescence of biological tissues, thus providing some additional chemical differentiation[1]. This correlation with biochemical

processes took a large step when immunostaining microscopy techniques were developed. This field has provided a valuable tool that is still quickly evolving—looking at the catalogue of the commercial supply house Molecular Probes (Eugene, OR) provides ample evidence of these advances. Growing out of static staining techniques is dynamic optical imaging, where new information about dynamics and localization of important bio-active agents can be discerned[2, 3]. For instance in one recent report, Qian & Kennedy developed a unique optical measurement of the release of Zn^{2+} based on confocal fluorescence microscopy in pancreatic β-cells in culture. With this novel method, colocalization of exocytotic release sites and Ca^{2+} entry was observed upon stimulation by glucose or K^+. However there are severe limitations with approaches rooted in traditional techniques[4]. These approaches require incubation of the tissue or cell with the fluorescent dye and thus rely on diffusion to transport the dye to the area of interest, which is often in fact not the case. Other techniques require the 'fixing' of the tissue, which often destroys cellular viability. There are also location-specific detection limitations defined by the transport of the diffusing dye: the resulting signal is a complex mixture of the concentration of the target and the specific transport mechanisms and kinetics associated with the dye transport and localization.

A technique that avoids the delivery of reagents to the cellular matrix is imaging mass spectrometry. From this technique, a rich amount of varied chemical information is available from fixed cellular tissues[5-9]. This class of methods extends far beyond that offered by traditional techniques since the mass spectrum for each sampling spot can conceivably be recorded. The techniques are centered on matrix assisted desorption ionization mass spectrometry (MALDI-MS), with new contributions coming from

desorption/ionization on porous silicon (DIOS) and direct imaging secondary ion mass spectrometry (DISIMS). The methods differ in how the appropriate amount of energy is added to the target molecules for desorption and ionization without their destruction. For MALDI-MS, the sample is co-precipitated with an organic matrix that adsorbs the laser energy and literally explodes the sample off the surface relatively in tact. DIOS is related to MALDI-MS, but the sample or tissue is deposited or grown on porous silicon, which can perform similar functions as the matrix in MALDI-MS. Both of these methods are well suited for analysis of high molecular weight biomolecules, whereas DISIMS uses a direct excitation of the sample with high-energy metal ions and is limited to low molecular weight targets since the ion beam destroys other materials[10-12]. With each of these methods, the tissue is either fixed or grown onto an appropriate substrate and a mass spectrum is recorded from a single spot from the resulting desorbed and ionized materials (Figure 2). The information from each location can be interpreted or the sampling spot can be rastered across the sample and an image is reconstructed from the resulting spectra. Even though the amount of chemical information available from these powerful new techniques is somewhat staggering, it is still limited in that no dynamics will be available from these techniques. To analyze the sample, it must be fixed and place in an appropriate vacuum chamber, extinguishing any biochemical activity.

Direct Probes

By fabricating very small direct probes-- that are literally small enough to be inserted into single cells or in very localized space in or about tissue--a variety of advantages for monitoring biochemical activity are realized. These small probes are

based on optical or electrochemical interrogation and are distinguished by transducing the concentration of a targeted biomolecule at the site of the probe tip to interpretable changes in the light or electric properties. These probes include microelectrodes, nanoprobes, nanosensors, or biosensors. The electrochemical transduction mechanism has been used to monitor fast changing localized concentrations of biomolecules that can easily undergo electron transfer reactions at modest electrochemical potentials—termed electroactive species. These probes can be extremely well localized (<1 μm to ~30 μm) and are a good match for high transient concentrations associated with cellular release or intracellular containment. The high fidelity measurements (both in terms of spatial and temporal resolution) available from microelectrodes has allowed monitoring of individual exocytic events where the changing concentration of neurotransmitter, hormone or neuropeptide can measured at the site of release and track the microsecond-time scale events[13]. The electrochemical probes can be operated in a potentiometric or amperometric form depending upon the specificity of the required measurement and the pre-existing knowledge of the tissue or cell being investigated. The chemical resolution of electrochemical measurements is limited to naturally electroactive species and generally no more than two species for a given potential sweep range.

Along the same lines as microelectrochemical probes is near-field fiber optic probes—called nanoprobes, nanosensors, and, if combined with some biological recognition, biosensors. These probes are able to generate highly localized light (~20- 50 nm in diameter) at the tip of a fiber optic cable by heating and pulling the tip to a very small diameter and depositing metal on the outer surface except exactly at the tip. This structure allows the penetration of some light even though the orifice is well below the

diameter of the diffraction limit. The light only penetrates the near field beyond the tip, so the volume being probed is extremely localized. This limited probe volume carries with it all of the advantages and disadvantages with the electrochemical probes, however, with some important differences in transport and detection issues. First, the biomolecule of interest must possess a unique spectral signature, either in an absorption wavelength with sufficiently small background to monitor altered concentration or fluorescence wavelengths (excitation/emission) that can be sufficiently correlated to a specific species. Alternatively, the probe tip can be modified with molecular/atomic recognition elements that change optical properties when interaction occurs. The other major difference is that most, if not all, of the target molecules are not consumed so that a depletion layer and the diffusion forces are not initiated. Thus, mass transport is generally not an issue for optical probes. Again, similar to electrochemical probes, there are only a limited subset of important bioactive molecules which possess an appropriate chromophore for selective detection in the complex milieu of living systems.

Microsampling

From a sampling point of view, all of these nanoprobes are dependent upon probe positioning and local diffusion for delivery of the target species to be interrogated. Thus, the sampled volumes are defined by the size of the probe and the characteristic diffusion distances and are typically in the picoliter to femtoliter range.

In contrast to on-site signal transduction, the removal and analysis of fluids from biological microenvironments has been accomplished with microdialysis probes; albeit with lower spatial resolution than the micro- and nanoprobes. This technique pumps fluid

pass a semipermeable membrane fabricated at the tip of a tubular probe, allows a limited range of species (based on molecular weight) to be sampled via trans-membrane diffusion, and collects the returning fluid, which is then analyzed for target species. The probe tip and adjoining structures are on the order of a millimeter and transport to the probe is generally based on diffusion so that the spatial resolution really is not in the ultrasmall volume regime, but this technique must be addressed for several reasons. It is closely related to newer approaches and is still commonly practiced in a variety of pharmacological and clinical settings. Also, it represents an important developmental step in the continuous sampling of living systems. This method has the distinct advantage of delivering a small sample to a remote location for standard biochemical or chemical analysis techniques. This is in stark contrast to the microscopy, electrochemical and near-field optical techniques that, in essence, transduce the analyte concentration to a spectral or electrical signal at the probe site. By delivering the sample to a remote site, a variety of analytes can be assessed by a number of methods, including immunoassay, separations, and mass spectrometry. With this combination of sampling and analysis, a wide variety of biomolecules and simultaneous multiple targets can be assessed.

Another very important factor that is unique to microdialysis approaches is the delivery of agents in the perfusion fluid. Pharmacological agents, hormones, neurotransmitters can be delivered to very localized tissues and the biochemical response can be monitored directly with the same apparatus. Certainly there are a number of limitations to this approach, where size is probably the most important: the amount of local tissue damage caused during insertion is significant, which not only decreases the spatial resolution, but can cause significant physiological artifacts.

Direct Sampling

Somewhere between microdialysis and nano- and microprobes is direct sampling. This technique removes a minute sample, transports it through a very narrow tube (sub-micron to 10s of microns) to be analyzed at a remote site[14-16]]. The sampled volumes can be on the order of picoliter to femtoliter, similar to that of other microprobes, but the fluid is actively transported some distance for remote analysis—consistent with microdialysis. This technique is based on the electrokinetic principles, the same ones which make capillary electrophoresis (CE) a high-resolution separation technique: efficient transport through narrow tubes (1-100 microns inner diameter), minimal dispersion of the original concentration profiles during transport, and a rigid, but flexible microtubing material. Clearly using pressure or vacuum on this size scales would not only be impractical, but possibly deadly to the tissue or animal under study. In addition, pressure-induced flow generates a significant amount of dispersion, which smears out the original concentration profiles and reduces the concentration through by dilution with the surrounding buffer. The detection systems must be compatible with the ultrasmall volumes and modest concentrations of some analytes and have therefore been limited to fluorescence, electrochemistry and mass spectrometry.

Capillary Electrophoresis and Electrokinetic Effects

The fundamental transport mechanisms of CE lend themselves to efficient separation science; CE has been directly applied to many biological problems. These same advantages of the electrokinetic effects have led to them being applied to many

microfluidic device transport problems. Since this technique a key part of microfluidics, some discussion of CE is warranted. Traditionally CE has been preformed in capillaries with inner dimensions of 15-75 µm[17]. Separations in smaller channel dimensions can improve the resolution and is one of the current goals for CE[18]. Wei and Shear performed analysis in channels with an inner diameter of approximately 600 nm and a sample volume of 180 fL in 1998[19]. More recently, the Ewing group was able to achieved separations of dopamine and catechol with sample volumes of 12 fL using 430 nm inner diameter tubing[17]. This work suggests the extremely high resolution in terms of volume, space and time are available with refinements in existing technologies.

Electroosmosis is the driving fluid movement force in traditional CE. Electroosmosis is the result between the interaction of the solution and the surface charge of the wall interface[20]. This fact is well shown by work from the Sandia National Laboratory graphically demonstrating the reduced dispersion when electroosmotic flow profile is compared with that of pressure induced flow (Figure 3) [21]. Since electroosmosis is an interaction of a surface to a liquid it has not been limited to capillaries and has been performed in microchannels as well [22].

Direct Sampling Combined with Microfluidic Devices.

A relatively new approach is to deliver the ultrasmall volume sample to a microchip device for purification, separation and analysis. This approach allows for various components to analyzed simultaneously while sampling an inconsequential amount of fluids from the system of interest. Importantly, ultrasmall volume bioanalysis can be performed in a variety of fashions on microfluidic devices. This is one method of

maximizing the information available from an ultrasmall volume sample that has been directly sampled. The rest of this chapter will focus on the development and application of microfluidic devices for small volume biological analysis.

Microfluidics on Microchip Devices

The field of microfluidics as an analytical focus of study can be traced back to the early 1990's. When Harrison, Ramsey, Manz and Widmer began fabricating microchips with fluid filled channels, they launched a new and exciting use for the well established fabrication methods for microelectronic and microelectromechanical technology[26]. The first chips, which were employed to separate small volume samples by electroosmosis, demonstrated the usefulness of the technique; however, the challenges of this type of system were recognized quickly. There was a need to develop chips with devices incorporated to controlled flow rates and enhanced separation efficiency.

Sample injection was an area that presented a challenge. In initial experiments, the problems of leakage and unpredictable transport were observed and were addressed in subsequent designs. For example, leakage of sample from one channel to another was found to be an extreme problem [27, 28]. Studies conducted to determine the extent of the problem showed that leakage could cause an increase of up to 20-30% of background fluorescence signal [29]. Higher applied potentials, used to control the flow and increase flow rate, caused higher leakage effects. So, although methods were developed to increase the rate of analysis and perform more complex functions by using more than one channel, they were creating problems with contamination and signal adulteration.

To combat this problem, pinched injection was introduced and became the method of choice[28, 30-32]. This is a method whereby the side channels are held under voltage control to induce a very slight backflow of solution, thus preventing the introduction of side-channel constituents into the main channel. (See Figure 4)

Once the primary problems of separation and injection were explored, the field was primed to move forward with analysis techniques. Capillary electrophoresis, chromatography[33, 34], mass spectrometry[35-37], bioassay[38, 39], DNA digestion and amplification[40-45], and even combinatorial synthesis reactions have been performed on a microchip system.

Work on improving the technology through increased reaction rates, separation times and better detection limits continues and has been greatly advanced to this point. Theoretical analysis of the components of microfluidics is also providing insight into new directions in fabrication and analysis [46, 47].

Much of the research in the 1990's centered on establishing fabrication methods and demonstrating the capabilities for the new technology. The use of electroosmotic pumps to control the flow rates of fluids provided a means for a valveless method of switching fluid flow through channels[22, 48]. The valveless switch has provided a means in which sample manipulation and capture can be preformed in volumes at and below microliters (Figure 5). During this time, continuous separation was accomplished by freeflow electrophoresis integrated on a silicon chip[49, 50]. This provided an improved "world-to-chip" interface, which had been and still remains a difficult issue[50]. Moreover, it provided a way for sample injection in a controlled manner at small volumes and low concentration[51].

The compact size of the microchip systems also allow for optimized space for large-scale analysis. A microfabricated chip with 48 lanes, each with the capacity to handle 2 samples, demonstrated the use of small volumes and fast analysis. All 96 samples were analyzed in less than 8 minutes [52]. Other means of increasing analysis rates such as increased efficiency and resolution with increased field strengths (for electrophoretic methods) and separation lengths have been successful as well[30, 53-56].

The advances in microfluidics on chips cannot be fully described without recognizing those who worked out the principles of preparing the microchip itself. Laser ablation of the microchip medium to produce channels and reservoirs opened the door to more complex reactions and analysis directly on the chip. Injection molded microfabricated electrophoretic separation devises were produced making another avenue to single use disposable chips a possibility[57]. Much of the research has focused on the fabrication of these microfluidic devises on planar glass[30]. Glass microchips performed far better than their silicon predecessors and exhibited better fluid control. Although glass has become the popular media in which fluid manipulation is preformed it has suffered from problems in bioanalysis.

Polymers

Polymers are being explored as another format for creating microfluidic devices. The growth in research using polymers as a material to fabricate microfludic devices on chips has grown considerably. The application of a polymer substrate provides several benefits compared to glass. The physical properties of the polymer must be taken into

consideration in fabricating these devices. Table 2 lists a number of characteristic that

need to be considered for polymer selection.

Ideal Polymer Properties

• Compatible with photolithographic developers	• Optically transparent to detection scheme
• Little or no autofluorescence	• Inert to experimental condition
• Surface can be modified	• High dielectric strength
• High thermal conductivity	

Table 2

Conventional methods of fabricating microfluidic devices in glass have mostly

required the fabrication process to be performed in a cleanroom [18] Polymer devices can

be created relatively fast using soft-lithographic techniques and have the benefit of

conceivably being inexpensive[58]. A number of different polymers have been used for

the creating microchannels are listed in Table 3.

	Glass transition temperature (K)	Dielectric strength (V/cm)	Thermal conductivity (Wm/K)	Tensile strength (psi)	Effect of strong bases, acids	Effect of organic solvents	Clarity (UV/vis)
Poly(methyl-methacrylate)	377	17.7×10^4	0.193	7000–11000	Attacked, acids only	Soluble	Opaque/ transparent
Poly(tetraflu-oroethylene)	> 523	15.7×10^4	0.405	2000–5000	Resistant below 80 °C	Very resistant	Opaque/ transparent
Poly(carbonate)	430	3800	0.193	8000–9500	Attacked	Soluble	Transparent/ transparent
Polybutene	249	NA	0.130	3800–4400	Attacked, acids only	Resistant	Opaque/ transparent
Poly(oxydimeth-ylsilylene)	265	NA	0.20	NA	Attacked, bases only	Soluble	Transparent/ transparent
Polystryene	381	5000–7000	0.116	5000–12000	Attacked, acids only	Soluble	Transparent/ transparent
Glass	>923	11.8×10^4	0.732	4000–8000	Resistant	Resistant	Transparent/ transparent
Silicon	>923	30.0×10^4	0.0149	16400	Resistant	Resistant	Opaque/ opaque

Table 3 [Values obtained from Polymer Handbook, 4th edition. J. Brandrup, E. H. Immergut, E.A. Grulke, Eds.Wiley–Interscience, New York, NY; 1999]

PDMS is an attractive polymer to work with for a number of reasons. PDMS is capable of reproducing surface features at the micron level[18]. It is also optically transparent to ~230 nm and exhibits a high electrical volume resistance. Furthermore, it can seal itself if punctured and a variety of methods exist to control its surface chemistry [18].

Channel Geometry

Channel designs have evolved alternative geometries demonstrating progress in designing complex systems for miniaturized total analysis systems (μ-TAS). Initially turn geometry was decided a poor choice where it reduced the efficiency of band shape[30]. The reduced efficiency was a consequence of the difference in path length of the inner and outer radius of the turn. Mathies reported on an optimization of turn geometry, trying a variety of different turns shapes; square, rounded, and tapered. (Figure 6) The system was tested by examining the resolution of the 271/281 base pair doublet in the separation of a φX174 *Hae*III DNA sizing ladder. The work resulted in two parameters that should be considered in optimizing new geometries. First, that, turns with the smallest radius of curvature minimize the effect of tilted lateral diffusion and, second, that the resolution per channel length improves as the radius of curvature is reduced and taper length increases[59].

Fabrication Techniques

New fabrication techniques were employed to bring a greater variety of materials control to the microfluidic systems[60, 61]. Hot embossing, injection molding, casting, laser ablation, and ion beam etching have all been employed for fabrication of microchannels. With the exception ion beam etching, all have come about as a fabrication technique for polymer microchannels in the last five years.

Hot embossing is currently the most widely used replication process for fabricating microchannels [58]. A relatively simply technique is heating a vacuum chamber to just above the glass transition temperature of the material, and applying a stamp with a hydraulic press to the material while it begins to cool. The stamp is then carefully removed, and the cycle is ready to begin again. Hot embossing has been demonstrated to create channels with less than a micron in width in PMMA[58], and a total fabrication time of less than 5 minutes[62]. Hot embossing minimizes replication errors due to the controlled nature of the pressure being applied to the stamp[62].

Injection molding is very similar to hot embossing, where its origins are traced back to the macroscopic world [58]. Polymer pellets are feed into a heated syringe to form a flowing polymer [62]. Then polymer is then injected by pressure onto the master mold, under vacuum, to aid in filling all spaces. This system differs from hot embossing in that it allows for an automated process, typically about 3 minutes, and was derived from industry [58].

Another alternative is casting, which is a much slower process for creating microchannels. The amount of time for the channel to be produced depends on the curing time for the polymer in use. While a much slower process, casting is a much simpler

technique that requires minimal capital investment [58], producing channels by pouring the polymer over the master mold and allowing them to cure [18].

Laser ablation allows for rapid fabrication of microchannels on a single device. While removing the need of a master mold, laser ablation does require a mask, an xy-table with a high degree of precision, and a laser [58]. The polymer is exposed to the laser, which then undergoes decomposition [62]. Depth control is limited to a tenth of a micron, however the surface chemistry is different when compared to the previous techniques due to the interaction of the laser light [58].

	Number of masks	Type of mask	Smooth walls, floor	3D Structures	Machine in glass, quartz	Machine in polymers
LIGA	2	Optical, X–ray	Yes	Yes	No	Yes
Laser ablation	1	Optical	No	No	No	Yes
Reactive ion etching	0	None	No	No	Yes	Yes
Imprinting	1	Optical	Yes	No	No	Yes
Molding	1 or 2	Optical, X–ray	Yes	No	No	Yes

Table 3-Properties of different fabrication techniques

Detection

There are several detection systems used on microfluidic devices, including: laser induced fluorescence (LIF), electrochemical (ECD), and mass spectrometry (MS). LIF has been argued to be the best-suited detection system[17]. Although chemical derivatization may be necessary, it can be performed post-separation in a controlled environment.

Electrochemical detection is useful tool for small volume detection. ECD is not limited by the reaction time restraints of performing post separation modification as with

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fluorescence. Furthermore, ECD is sensitive to mass, instead of volume. Specifically, amperometric detections are appealing because they are highly sensitive, selective, and relatively low cost to setup and maintain[69].

Existing technologies were adapted to a microchip device with a mass spectrometer. Electrospray created by an electric field at the side of the chip provided the necessary means transfer effluent to a mass spectrometer[36, 37]. With electrospray ionization, the components separated by CE are introduced into the mass spectrometry.

Biology

For ultrasmall volume bioanalysis, some would consider the DNA and immunoassay arrays within this category. In fact, by 1997 the lab-on-a-chip concept had its first commercially available products within this category. Substrate separation, immunoassay, DNA digestion and amplification, and analysis of human serum proteins had been accomplished in published systems[40-45]. However, these systems have not been coupled to any ultrasmall volume or single cell analysis schemes nor are any dynamic information obtained from these devices, and, hence, are somewhat beyond the scope of this chapter [38, 39, 70, 71].

Microfluidic devices can be exploited by either directly growing or transferring cultured or extracted cells, or tissues to the device for cellular manipulation and analysis. Assessing the properties of cells by flow cytometry is a valuable mechanism to investigate populations of cells either transferred to the microdevice or grown within a portion of the device. Analysis of cells has typically been accomplished in bulk by taking a large homogeneous sample, processing the material, and performing a traditional

biochemical or chemical analysis. These methods have been, of course, a valuable paradigm for clinical applications such as finding the average amount of hemoglobin in a cell[23]. Obviously, this method cannot be easily transferred to ultrasmall volumes.

Single cell analysis can provide more intimate information about the cellular function and its communication with its environment. Rather than looking at the bulk sample of cells and extrapolating information about activity to a single cells, as has been the traditional approach, a large number of single cell measurements can be performed which in turn can both provide detailed individual cellular information and provide a much more detailed statistical basis for cell populations. Single cell analysis has the potential for generating an early detection system of cell damage or abnormality [24]. However, it is difficult to detect a few cells with irregularities among a large population of healthy cells.

Recently, the sensitivity of bulk cell analysis was compared to that of single cell analysis using human colon adenocarcinoma cells or HT29. Figure 7A shows the separation of the components of a cell by capillary electrophoresis, where as 7B shows a similar analysis of a bulk sample[25]. Figure 7B illustrates an averaged peak of the total cell population. Does the single cell represent a sub-population or specific exceptional case or is it within the statistical norm of the distribution of normal or bulk-sampled cells? This question can be adequately addressed with multiple single cell analyses whereas traditional techniques fall short.

Importantly, for application of microfluidic devices to small volume sampling cell and protein adhesion can become a problem due to debris left on the channel walls[72]. During an initial exposure, the cell generally does not adhere nor does debris obstruct the

channel, but subsequent exposure leads to clogging if sufficient cleaning procedures are not followed[73]. Debris on the channel walls can influence the accuracy of transport and analysis. Decreasing the channel dimensions has resulted in a better retention efficiency where shear stresses apparently play a large role in cellular transport[72].

While using small channel sizes can minimize effects of adhered cellular debris, it does not eliminate the problem entirely. As an alternative, coating the channel wall has been one way of minimizing the adhesion of cells. To measure the extent of cell adhesion, *E. coli* has been used because of their tendency to affix to glass. Alternatively, PDMA demonstrated minimal adhesion, but generates an unwanted consequence of suppressing electroosmotic flow. Channels coated with PDMA had bovine serum albumin mixed with the buffer to eliminate any remaining adhesion problems[74].

One challenge of performing CE analysis is cell injection. Electrokinetic and hydrodynamic injection systems have been used depending on the experimental condition. Electrokinetic injections are typically used for cells that adhere to surfaces, and when trying to introduce the sample to a MS system [75]. An injection time of 1 second is enough time to provide an adequate injection when a potential of –1 kV [17]. The other traditional method of injecting a cell into a capillary is by hydrodynamics. A hydrodynamic injection works by creating a suction that can hold onto the cell that is suspended in solution. A third technique for performing single cell injection is optical trapping of a cell[51]. One limitation, or advantage, depending on the specific circumstance with these methods is that they are limited to a single injection and separation at a time[24].

Once a cell has been introduced into the channel, cell lysis can be induced within the first minute of the separation, typically. Cell debris can and often does create a problem with sticking and/or blocking the channel due to the small channel dimensions [18], and as a consequence the channel must be cleaned in between each run or run the risk of affecting further CE run [25]. Dovichi devised an automated way of performing single cell analysis while looking at glycosylation and glycolysis from a single human carcinoma cell. While the technique was somewhat limited, it demonstrated a cell monitoring system during injection. Once introduced to the capillary, the cell could be lysed with 30 seconds of injection, a separation preformed, and then a reconditioning of the separation capillary[25].

While Dovichi's work increased the speed in which analysis could be preformed, it still was not an optimized system for two reasons. First, that manual observation of cell injection was needed. This proves to be tricky when trying to keep the total injection length below 400 μm. Second, that the system wasn't efficient because of down time while the capillary was rinsed.

Chen and Lillard devised a system that allowed for repeated whole cell injections while at the same time decreasing the amount of cell debris entering into the capillary. They introduced a lysis zone by introduction a gap junction. The exit of the cell injection capillary is ~5 μm away from the injection entrance of the separation capillary. This allows the cell to flow into the junction and then undergo lysis without the use of a detergent, where the cellular contents flow into the separation capillary. The separation of carbonic anhydrase II and hemoglobin from a human erythrocyte sample were

demonstrated (Figures 8 and 9)[24], which illustrates how rapidly multiple cell runs can be preformed and the unique nature of each cell.

In addition to performing separations, electrokinetic effects demonstrate that they can effectively control flow dynamics. Work by Harrison demonstrates sample manipulation, on a microchip, of bakers yeast, E. Coli, and canine erythrocytes (figures 4 and 5) [72]. While differing in shape and size, all of these cells flow along the electric field lines by electrokinetic effects (electroosmosis and electrophoresis). In addition to controlling the flow of the cells, controlled chemical reactions can be induced. For example, the labeling of proteins and cell lysing can be done on chip in a controllable manner using a valveless format by changing the electric potential at the junction [72]. In addition, mixing can be performed in these systems [76].

Biological Application and Detection

Early work out of the Ewing group has led to a variety of interesting applications of CE in monitoring single cell release[14-16]. One recent and particularly interesting is monitoring of the cerebral ganglion neurons of *Aplysia Californica[77]*. Although the experimental parameters for the study of neural cells is not conducted under conditions encountered in nature, it is developing the methodology for the future experimentation. Probing *Aplysia Californica* with CE/MALDI MS allows for isolation of proteins and peptides in a manner that allows for further study, due to samples being deposited onto a MALDI plate[78]. By coupling CE with MS, two different separation mechanisms (CE charge to drag, and MS charge to mass) are coupled together to obtain highly resolved temporal and special data, which is ideal for studying complex biological systems[79].

Such data can begin to give insight about how the cellular components are arranged

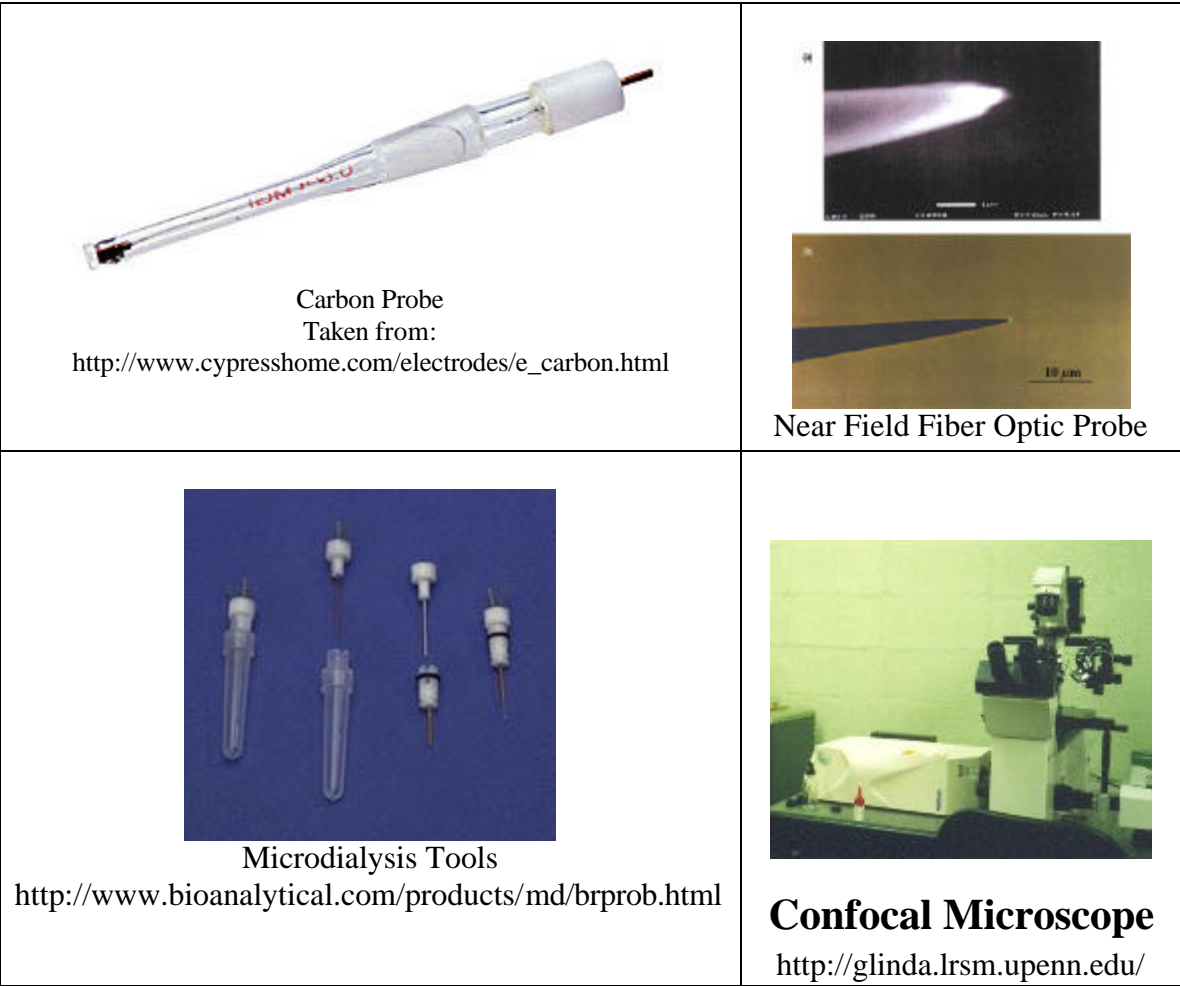within the cell and how its function varies to adjacent cells.

Carbon Probe Taken from: http://www.cypresshome.com/electrodes/e_carbon.html	Near Field Fiber Optic Probe
Microdialysis Tools http://www.bioanalytical.com/products/md/brprob.html	**Confocal Microscope** http://glinda.lrsm.upenn.edu/

Figure 1
Types of probes used for studying biology
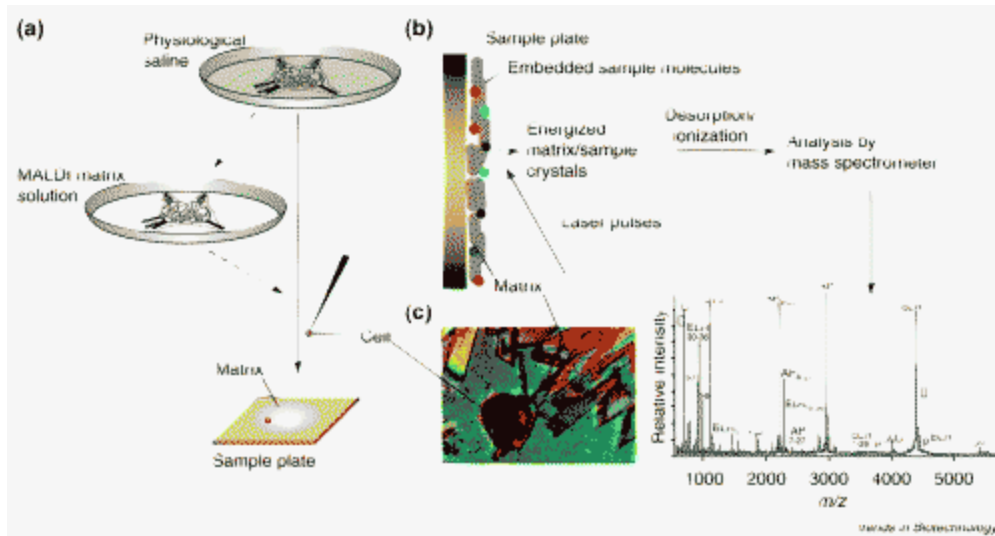
Figure 2. Overview of single-cell MALDI-MS. **(a)** The strategy of cell isolation and sample preparation. Briefly, following animal dissection, the tissue or ganglion of interest is pinned down in a dissection dish. Individual cells are then isolated either in the presence of physiological saline (freshwater specimens) or within a solution of MALDI matrix (marine specimens). Most cells are subsequently placed onto a MALDI sample plate containing a drop of matrix and allowed to air dry. **(b)** The MALDI-ionization process. In MALDI-MS, the analyte is dilutely embedded in a solid matrix and bombarded with ultraviolet or infrared laser pulses. The laser causes an explosion-like ion plume, which desorbs and ionizes the compounds in the sample into the gas phase, and the ions are mass analysed with a time-of-flight mass analyser. Shown is a typical mass spectrum from a single bag cell neuron from *Aplysia californica*. **(c)** A cultured neuron with cellular processes and matrix crystals visible.[Li, 2000 #63]
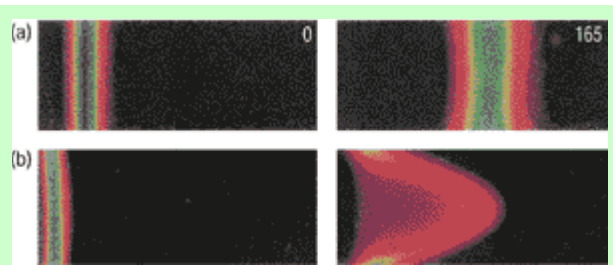
Figure 3. Electrokinetic transport versus pressure-driven flow.
(a) Electrokinetic flow was achieved through a 75-μm i.d. capillary at a 200 V/cm potential field strength. (b) Pressure-driven flow was achieved through a 100-μm i.d. fused-silica capillary (5 cm of H_2O per 60 cm of column length). Images show a caged fluorescein dextran dye moving under both flow mechanisms at $t = 0$ and at $t = 165$ ms.
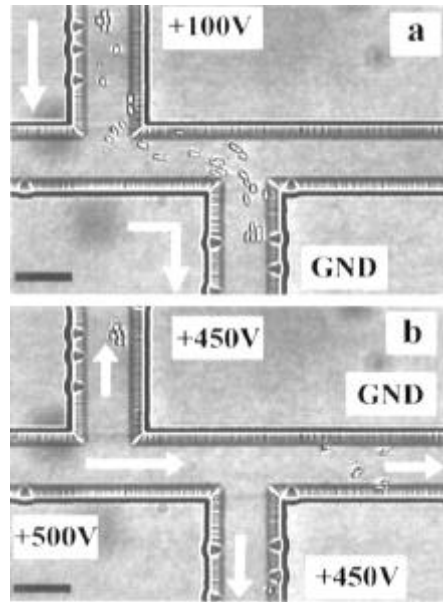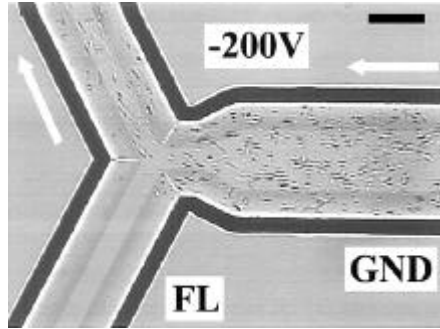
Figure 4- A microfluidic device pinched injection of *E. coli* cells

Figure 5

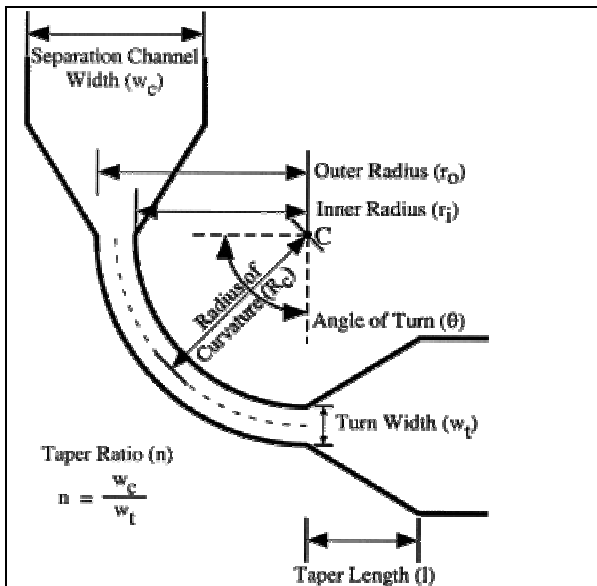The path of flow can be changed from the top channel to the bottom with the valveless

switch.

Figure 6A Defining parameters of a tapered turn in a microfabricated channel. The width of the channel in the turn is denoted w_t and the width in the separation channel is defined as w_c. The turn subtends an arc of θ rad with a radius of curvature R_c. The width, w_t, can also be defined in terms of the inner and outer radii of the channel, r_i and r_o, respectively.
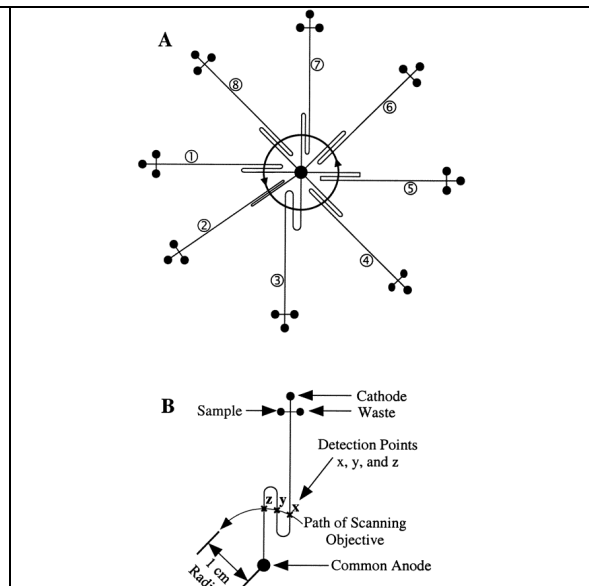

Figure 6B (A) Layout of the microplate. The 1-cm-radius path of the scanning objective is indicated by the arrow. The parameters of the turns in each channel are summarized in Table 1. (B) Magnified view of a single channel. The sample, waste, and cathode reservoirs were fabricated 3 mm from the injection cross. The 14-μm-deep separation channels were 138 μm wide and the injection cross-channels were 44 μm wide. The scanning objective monitored the separation at points x, y, and z placed 2.75, 3.82, and 4.93 cm, respectively from the injection cross.
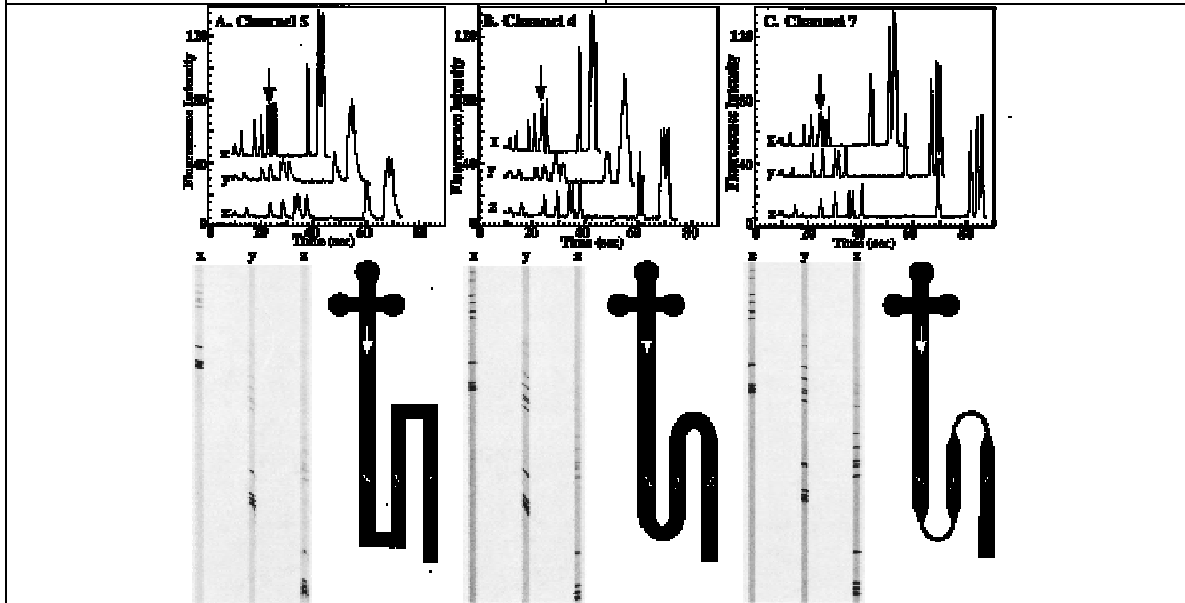

Figure 6C Electropherograms measured at detection points x, y, and z for channels 5, 4, and 7. An image of the separation and the channel design are given below each set of electropherograms. The square turn, channel 5., the U-shaped turn, channel 4. and the tapered turn with $R_c = 500$ μm, $l = 200$ μm, and $n = 4:1$, channel 7. The fragments of the ϕX174 *Hae*III digest DNA ladder are of lengths 72, 118, 194, 234, 271, 281, 310, 603, 872, 1078, and 1353 base pairs. The 271- and 218-bp peaks are indicated by the arrow. The channel designs are not drawn to scale.
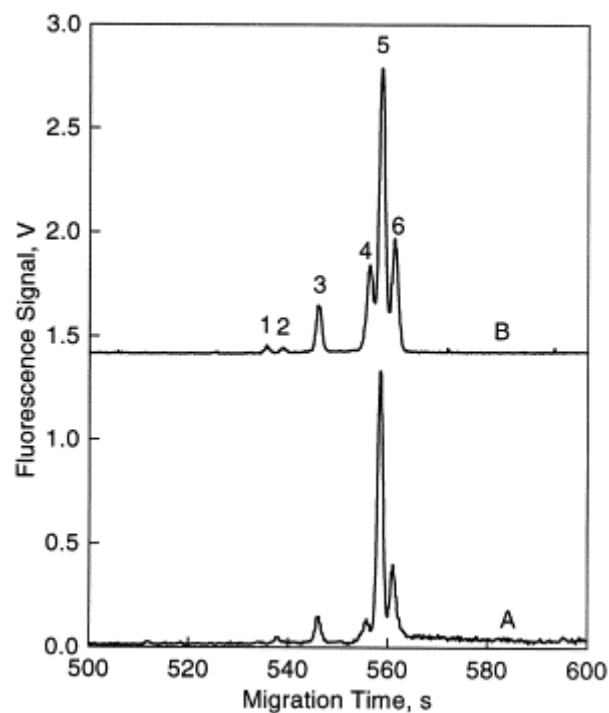
Figure 7. **Electropherograms obtained from (A) single-cell and (B) bulk analysis of the cell extract. The peaks correspond to: (1) a tetrasaccharide, Le^y- TMR; (2) a trisaccharide, Le^x- TMR; (3) unreacted substrate, LacNAc- TMR; (4) unidentified product; (5) a monosaccharide, βGlcNAc- TMR; (6) a TMR- aglycone, HO(CH$_2$)$_8$CONH(CH$_2$)$_2$NH- TMR.**
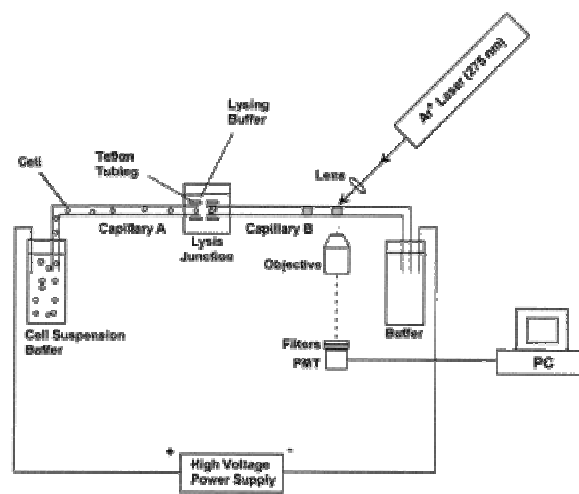
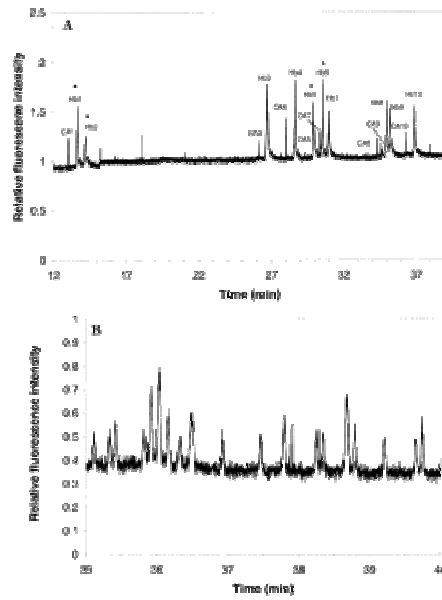Figure 8 Schematic of instrumental setup for high-throughput CE analysis of single cells.

Figure 9 shows a high-throughput analysis of single human erythrocytes preformed by a continuous single cell injection(A) and with 0.3%SDS in the lysis junction(B).

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