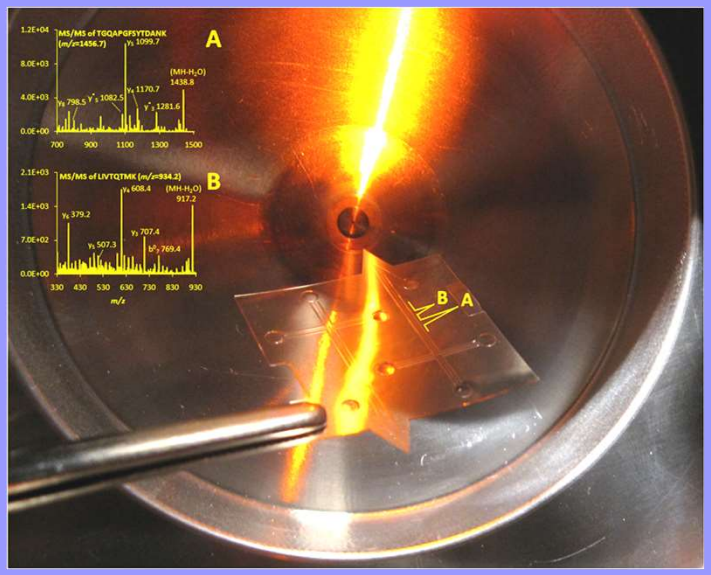

CHEM-E8135 Microfluidics and BioMEMS (5 cr)

10 March 2021

Microfluidic separation systems

for small (organic) molecules, peptides and proteins

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Brief history of Micro Total Analysis Systems

Early 1990's:

What if I put the whole Lab on a Chip?

Andreas Manz
https://www.youtube.com/watch?v=lLiGhFzs4sw

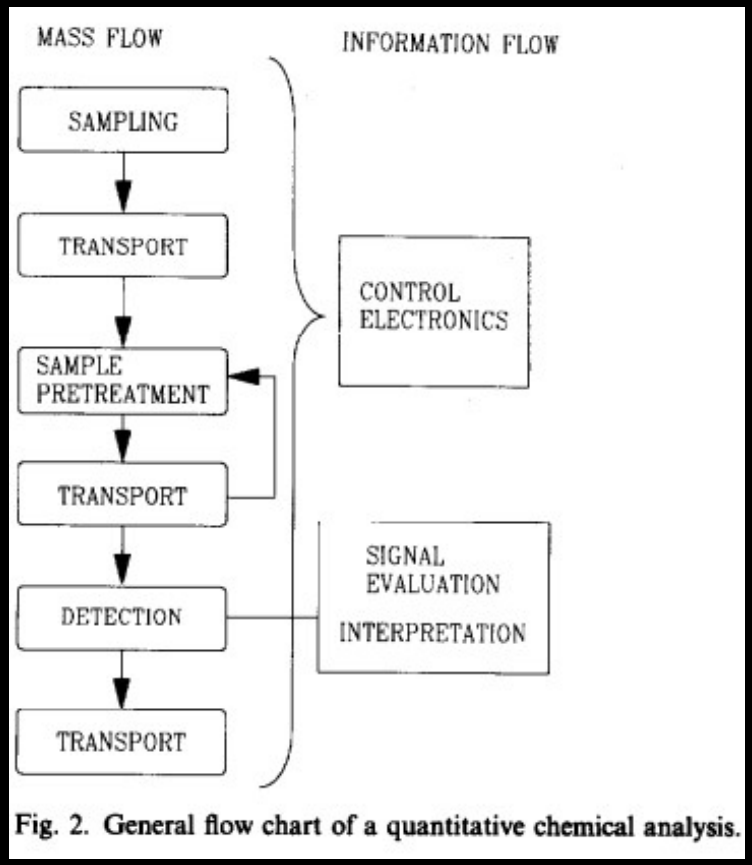


Fig. 2. General flow chart of a quantitative chemical analysis.

Manz, A., et al., Sensors Actuators B1 ***1990****, 244-248.*

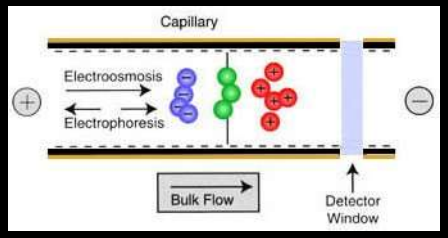
Originally developed for and refered to chemical analysis of small molecules.

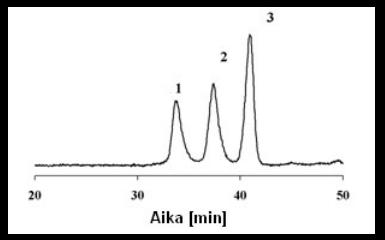
Micro Total Analysis Systems → Lab-on-a-chip

Early 1990's:

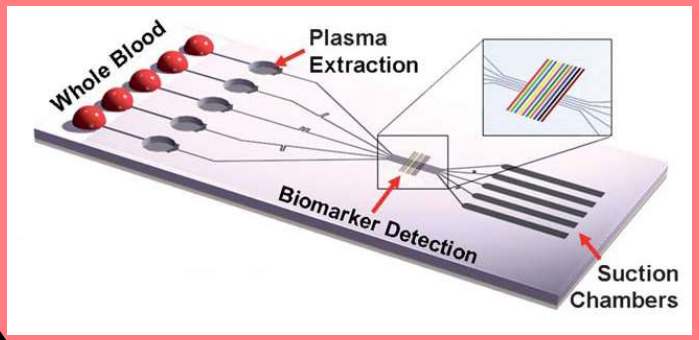
What if I put the whole Lab on a Chip?

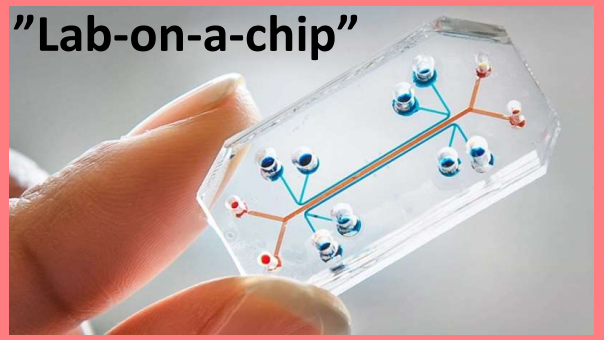
Andreas Manz
https://www.youtube.com/watch?v=lLiGhFzs4sw





Sample Preparation → Sample Loading → Separation of Components → Detection of Components → Readout





Microfabrication Technology – The Key Facilitator

A typical process flow for a glass microchip

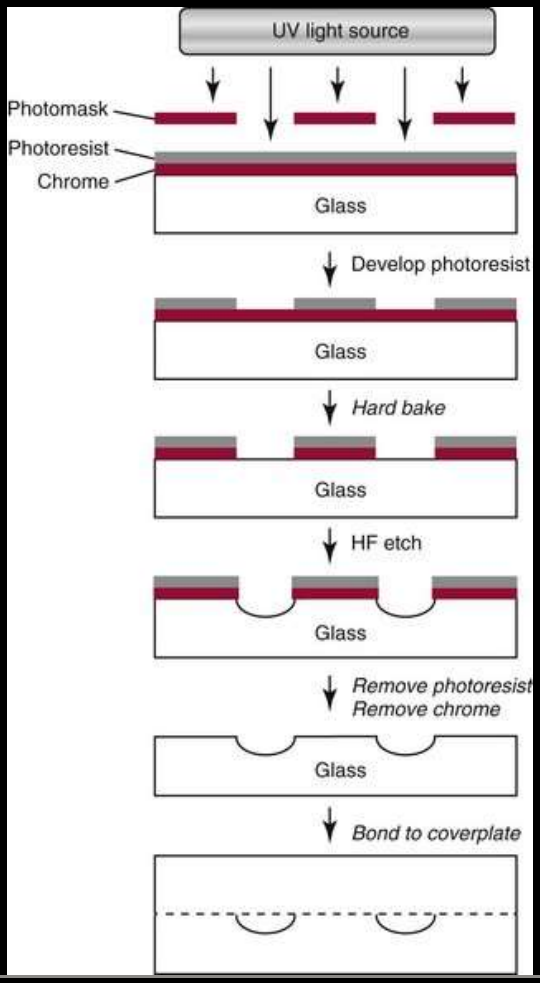
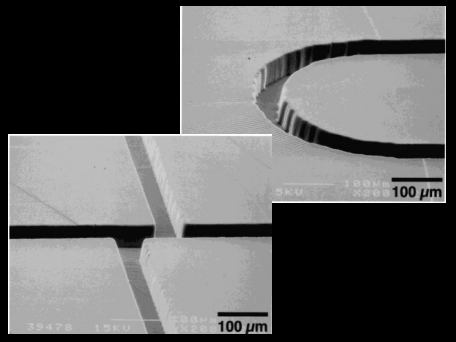


Figure from: https://basicmedicalkey.com/microfabrication-and-microfluidics-and-their-application-to-clinical-diagnostics/

The key benefits of PDMS = polydimethyl siloxane

- inherently biocompatible & gas-permeable
- bonds (self-adheres) reversibly to almost any material
- heat-curable
- reproduces the shapes of the (positive) photoresist relief upon molding 1:1
- abundant & inexpensive



*Duffy, D.C., McDonald, J.C., Schueller, O.J.A., and **Whitesides, G.M.**, Anal. Chem. 1998, 70, 4974.*

*https://www.ted.com/talks/george_whitesides_**toward_a_science_of_simplicity***

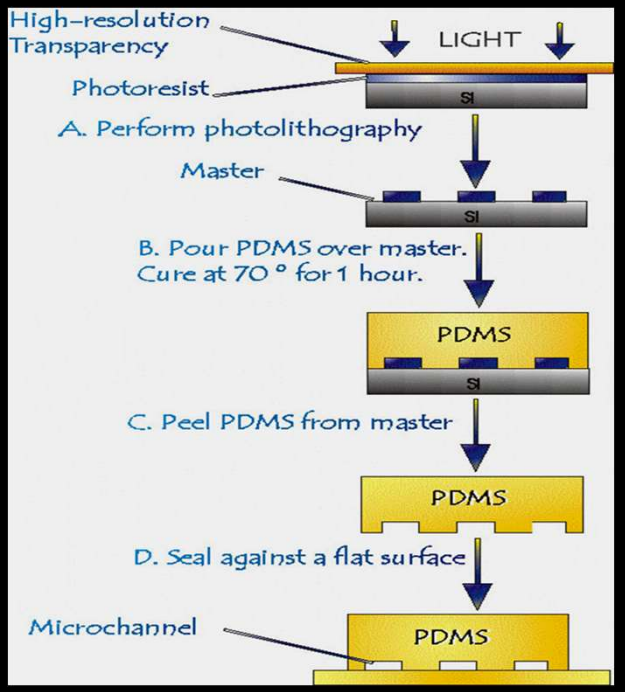


*Figure from: Huikko, K., et al., Lab Chip **2003**, 3, 67.*

Microfluidics in chemistry and biology

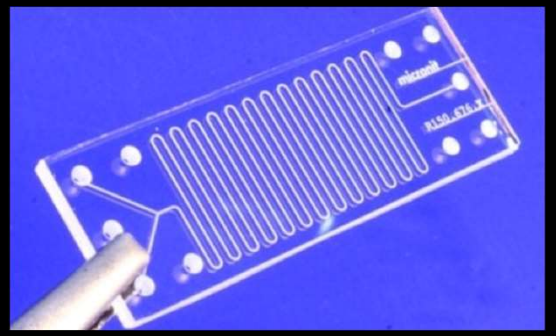
= Controlled actuation of minute (pL-nL) sample volumes in μm-scale structures

⇒ **Precise dosing**
⇒ **Fine spatial & temporal chemical gradients**

I. Micro Total Analysis Systems (miniaturized separation systems)
- Rapid analysis, low reagent consumption, less waste

II. Organ-on-a-chips: 2D and 3D cell models
- Controlled supply of nutrients, oxygen, drugs, toxicants
- Under evaluation for drug discovery by FDA

III. Microreactor technology for ”sample preparation”
- Rapid purifications, labelings etc.
- Enzymatic & nonenzymatic reactions (with immobilized catalysts)

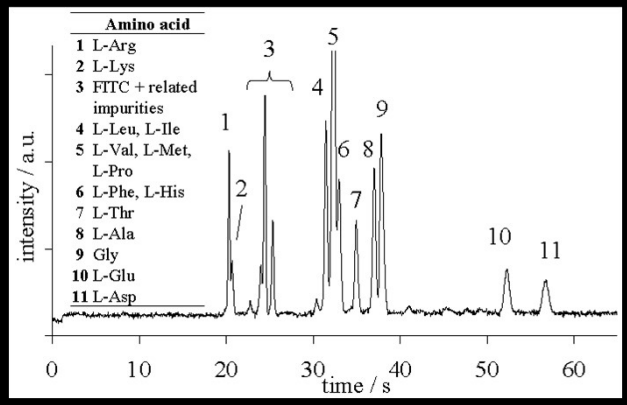


Why miniaturize the separation systems?

- **The technical advantages of Micro Total Analysis Systems**
  - Integrated unit operations → negligible “dead” volume
    → very rapid analysis
  - Minute total volume → low reagent consumption

Picture:
https://www.gene-quantification.de/lab-on-chip.html

- **Microchip (zone) electrophoresis = the gold standard**
  - Straightforward to miniaturise, very rapid analysis (~1 min)
  - Can be integrated with a range of detectors: optical, electrochemical, mass spectrometry
  - Requires electrically insulating manufacturing materials (e.g., glass)

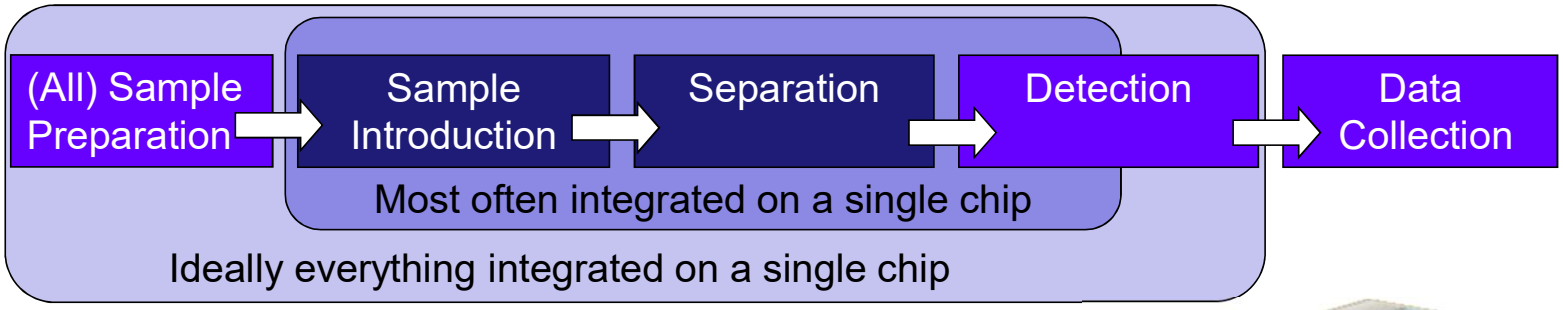


Learning objectives

- To understand which are:
  - The main separation techniques feasible for miniaturization of small molecule (incl. peptides and proteins) analysis and which are the key components of the related instrumentation
    - Electrophoresis on chip
    - Liquid chromatography on chip
  - The main detection techniques used in miniaturized separation devices
    - Optical, electrochemical, and mass spectrometric detection
  - The main benefits and hurdles of miniaturization of separation systems

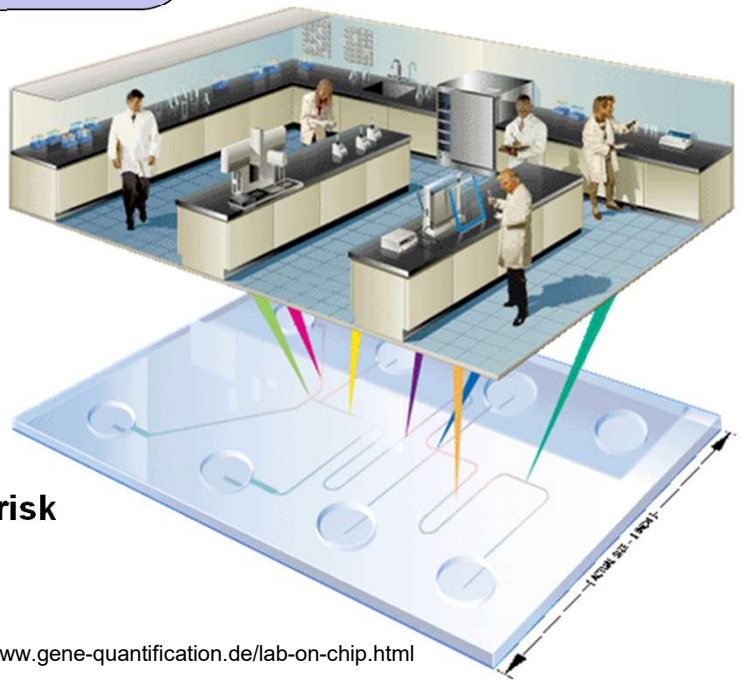
Chemical Analysis

Micro Total Analysis Systems (μTAS) ≈ Lab-on-a-Chip



- **DRIVING FORCE**
  - great demand for fast analyses and higher throughput methods among bioanalysis, pharmaceutical industry, clinical analysis, environmental monitoring…

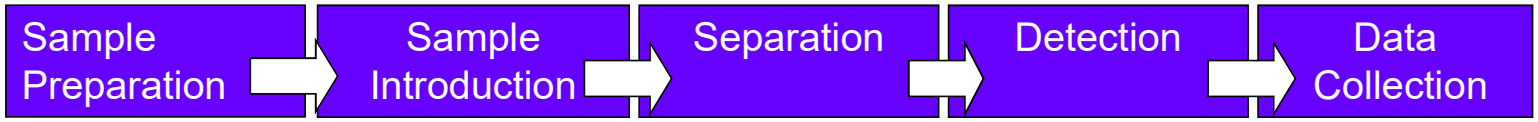
- **WHY MINIATURIZE? - THE USER ADVANTAGES**
  - integration of unit operation on a single chip
    - zero dead volume → **no time lag**
    - minute sample volumes → **low cost, less waste**
  - parallel devices → **multiplies the throughput = fast!**
  - mass production of disposable devices → **no cross-contamination risk**

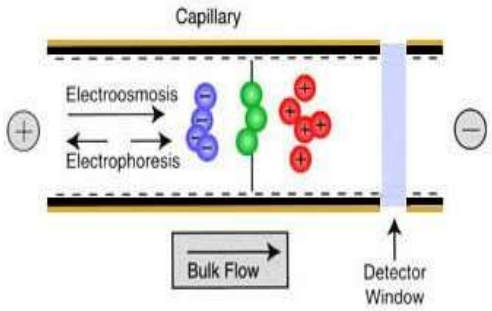


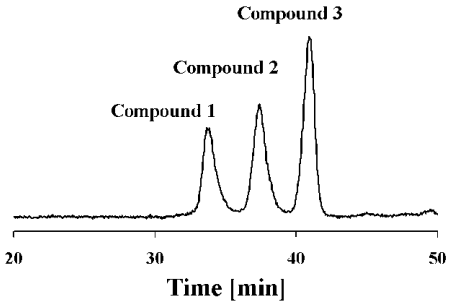
Picture: https://www.gene-quantification.de/lab-on-chip.html

Chemical Analysis

Common unit operations of instrumental analysis







Most widely used methods in (bio)chemical analysis:

FOR SEPARATION:

*** liquid chromatography (LC)**

* gas chromatography (GC)

* **electrophoretic separation techniques**

(CZE, IEF, MEKC, gel electrophoresis…)

FOR DETECTION:

** optical detection (UV, fluorescence…)

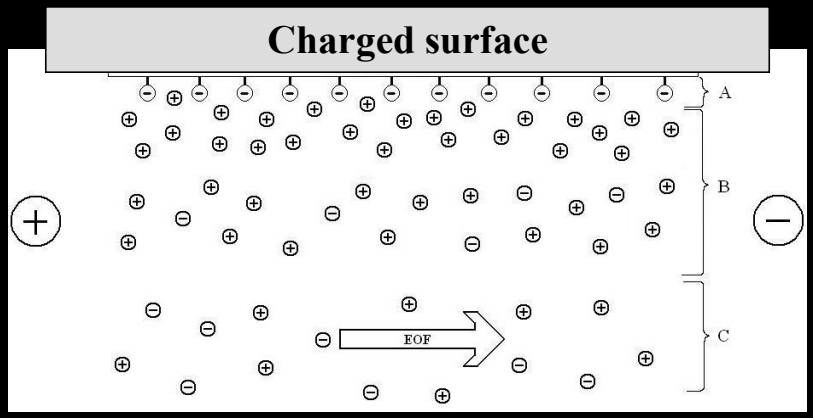
** mass spectrometry

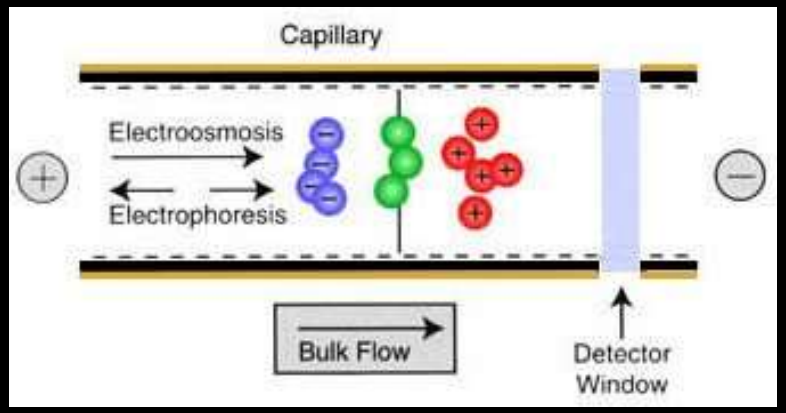
** electrochemical, radioactive…

Electrophoretic separations on chip

Capillary (free zone) electrophoresis = CE

- Electric-field driven movement of electrolyte ions (bulk flow) → Electroosmotic flow (EOF)
- Electric-field driven separation of analytes based on size and charge → Electrophoresis





The linear velocity is dependent on the electric field strength

In microchannels:

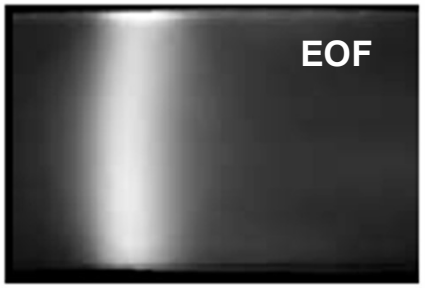
- applied electric field strengths typically hundreds of V/cm
- electroosmosis → typically $\mu_{EO}=10^{-4}$ $m^2/(V\times s)$ → linear v=0,5-1 mm/s
- electrophoresis → typically $\mu_{EP}=10^{-6}\ldots10^{-5}$ $cm^2/(V\times s)$ → linear v=0,001…0,1 mm/s

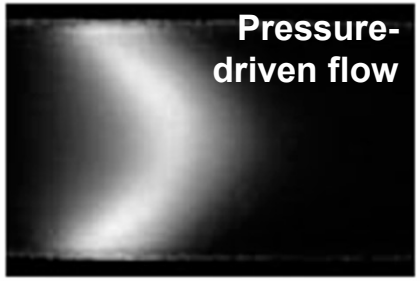
Electroosmotic flow (EOF) = Electric-field driven bulk flow

Laminar flow & plug-like flow profile

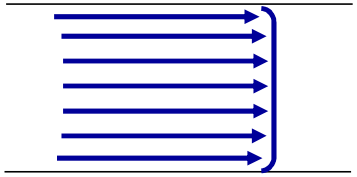
Microfluidic separation systems

sample zone profiles:



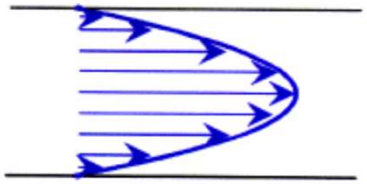


plug-like profile (EOF)



vs.

parabolic profile
(pressure induced flow)

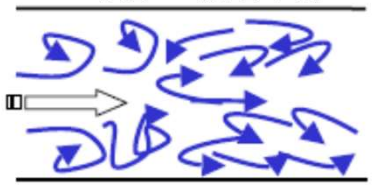


laminar flow
well-defined streamlines
(velocity of a given point does not vary with time)

Reynolds number (Re)

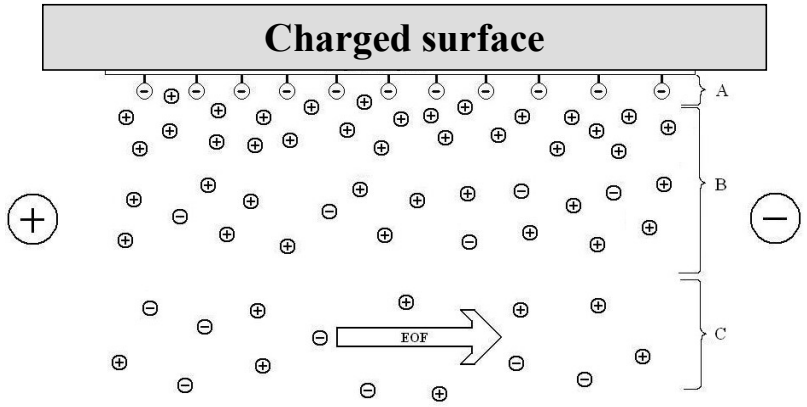
used for discrimination between laminar and turbulent flow

$$Re = \rho v d / \eta$$



turbulent flow
unstable streamlines

Birth of electroosmotic flow (EOF)



- formation of electrical double layer (EDL) on a charged surface by electrostatic attraction of counterions (Stern's model):
  - (A) a rigid double layer (Helmholtz layer) = immobile
  - (B) a diffuse double layer (Debye-Hückel layer) = mobile
    - 100 mM electrolyte solution → thickness ~0.5-1 nm (slower)
    - 1 mM electrolyte solution → thickness ~50-100 nm (faster)

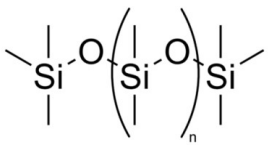
- velocity of EOF depends on the surface charge
  - "quantified" by zetapotential (ζ) between A and C [$\pm$ mV]

Surface charge on apparently neutral polymers

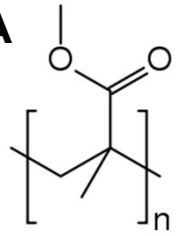
(Beattie, J.K., Lab Chip 2006, 6, 1409-1411.)

- Also neutral polymer surfaces often exhibit extensive EOF despite the lack of functional/chargeable groups (such as –Si-OH, -COOH...)

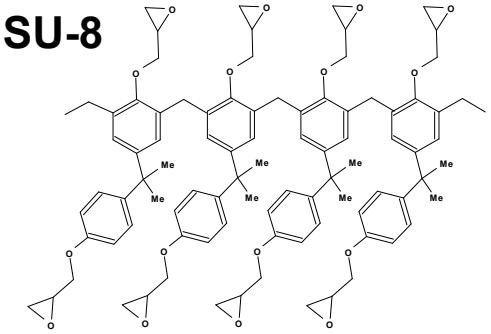
PDMS



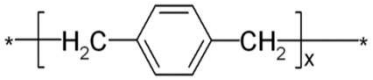
PMMA



SU-8



Parylene



- Experimentally determined zetapotential typically lie between
  - glass -20…-50 mV → cathodic EOF ~4…7×10^{-8} m^2/(Vs)
  - polymers 0…-20 mV → cathodic EOF ~0…4×10^{-8} m^2/(Vs)
- Correspond to
  - linear velocities ≤ 1 mm/s depends on electric field strength
  - volume flow low nL/min depends on channel cross-section size

Extra material: Surface Charge Determination

Several experimental methods available for zetapotential determination

$$\mu_{EOF} = \frac{v}{E} = \frac{L}{E \cdot t} = \frac{\varepsilon \cdot \zeta}{\eta}$$

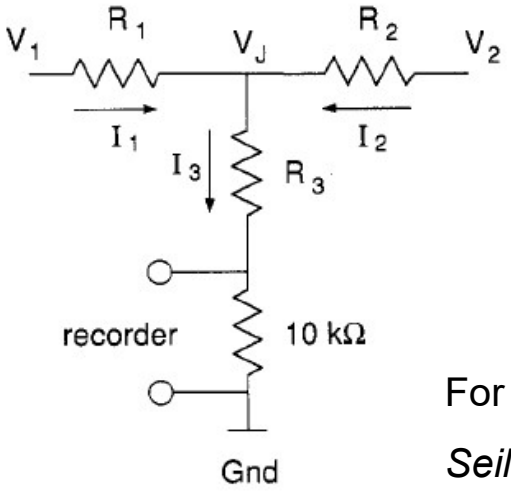
μ_{EOF}	electroosmotic mobility [m^2/Vs]
$v=L/t$	linear velocity (v)
E	electric field strength
ε	dielectric constant of solution
η	solution viscosity

- direct zetapotential (ζ) measurement
  - *nano-/microparticle mobility under applied electric field*
  - *Dynamic Light Scattering a.k.a. Photon Correlation Spectroscopy*
- direct EOF measurement (by fluorescence detection, for example)
  - *monitoring of the velocity of a neutral marker migrating along with EOF*
- current monitoring method
  - *measurement of a current drop along with decreasing buffer conductivity*
- streaming current, streaming potential
  - *induced by transport of EDL counterions under pressure-driven flow*
  - *"opposite" to electroosmotic flow, i.e., flow induces potential*

Valving of electrosomotic flow direction: Analogy to electrical circuits

- electrical resistance can be controlled by
  - microchannel dimensions (equivalent to conducting wire dimensions)
  - buffer conductivity (equivalent to conducting wire conductivity/resistivity)

- equivalent circuit for intersection of three channels with controlled potentials



$U = RI$

$R = \rho L / A$

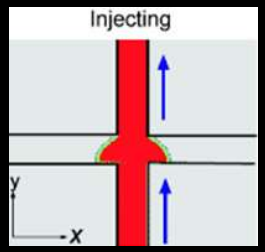
For more details, see:

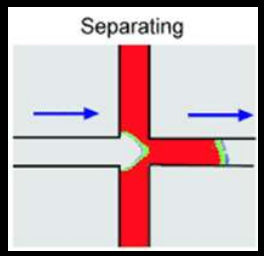
Seiler, K., et al., Anal. Chem. 1994,615, 3485-3491

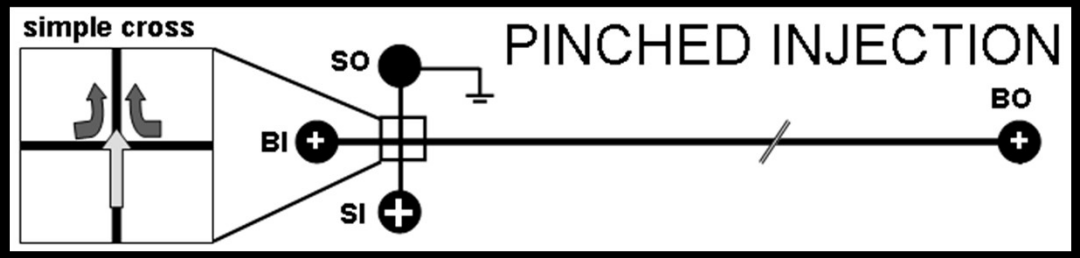
Microchip (free zone) electrophoresis

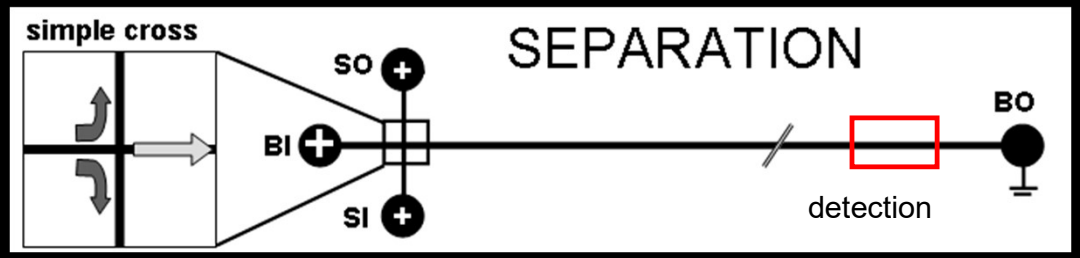
- Charged surface (originally glass, nowadays a lot of polymers too as manufacturing material)

- Sample loading into the system is done <u>across</u> the separation channel
  - Very narrow initial sample plug lentght (50-100 μm)
  - Thus, very rapid separation → Short separation path → High E-field with low P input







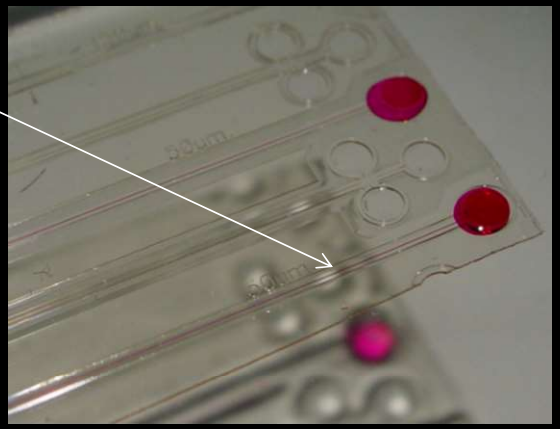


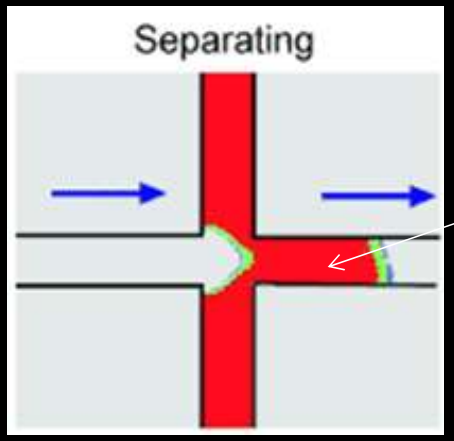
Figures from: T. Sikanen, Doctoral dissertation, University of Helsinki, 2007 & Encyclopedia of Microfluidics and Nanofluidics, Springer, 2008.

Microchip (free zone) electrophoresis (2)

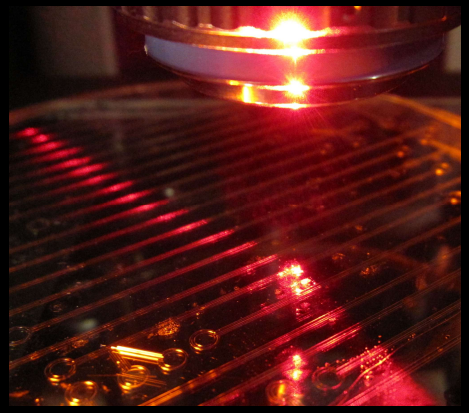
Microchannels

- cross diameter ~50 µm
- length ~few centimeters
- total volume 50-100 nL





Effective injected sample volume ~50-100 pL → very short separation path and time required



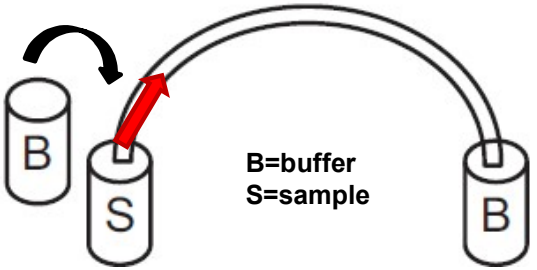
Separated components can be detected using fluorescence microscope (fluorogenic probes), or the chips can be combined with, e.g., mass spectrometer or electrochemical detectors

Figures from: Courtecy of T. Sikanen & Encyclopedia of Microfluidics and Nanofluidics, Springer, 2008.

Sample introduction protocols

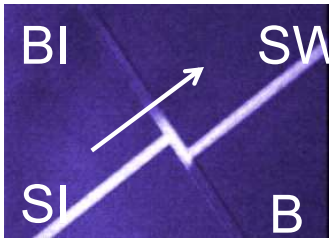
Sample introduction in conventional CE (in capillaries)

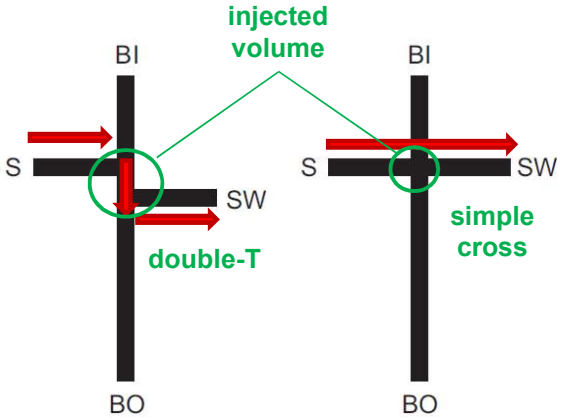
- Voltage- or pressure-driven
- Injection volume dependent on time



Sample introduction on microchips

- Nearly always voltage-driven
- Always across the separation channel
  - No charge discrimination
  - Injection volume dependent on cross-section dimensions (simple cross vs. double-T)





BI=buffer inlet, BO=buffer outlet, SI=sample inlet, SW=sample waste

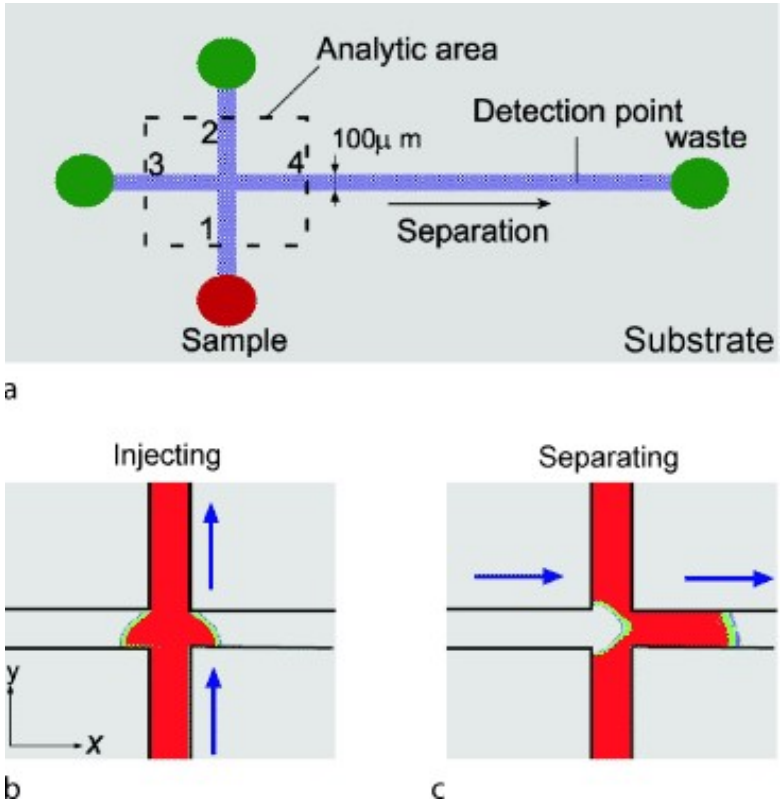
Sample introduction on microchips

Injection in ”floating” mode

- Injection step:
  voltage applied between sample inlet and sample waste only

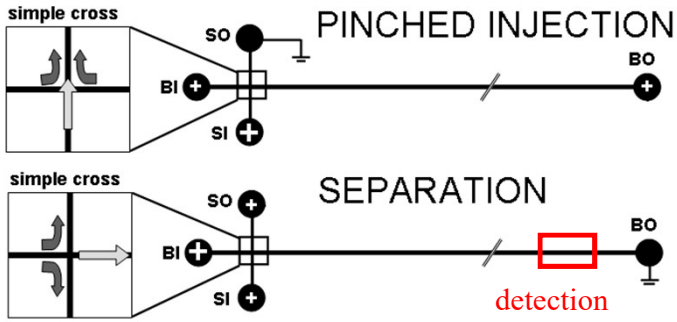
- Separation step:
  voltage applied between buffer inlet and buffer outlet only

- May be prone to hydrostatic pressure effects causing sample leakage

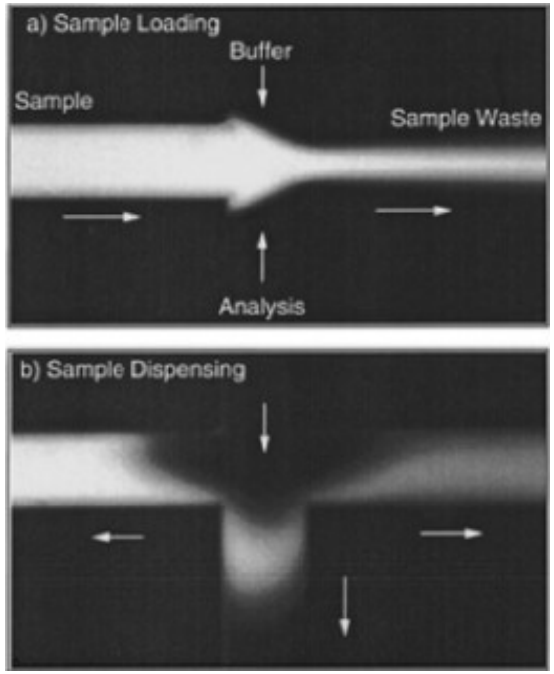


Schematic from Encyclopedia of Microfluidics and Nanofluidics, Springer, **2008**.

Sample introduction on microchips (2)



Schematic from T. Sikanen, Doctoral dissertation, University of Helsinki, **2007**.



Wu, D., et al., *J. Chromatogr. A* ***2008,*** 1184, 542.

Injection in ”pinched” mode

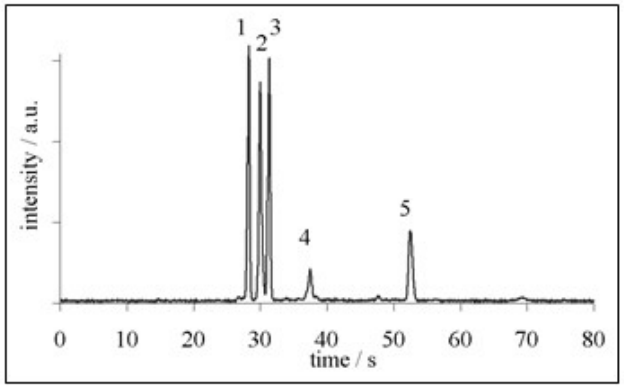
- Injection step:
  pinching voltages applied to buffer inlet and outlet

- Separation step:
  pushback voltages applied to sample inlet and waste

- Better control over injected sample volume and leakage over floating mode

Some Characteristic Performance Parameters

- migration time (t_{migr})
  - the appearance time calculated from the end of the injection step, (i.e., separation step starts at t=0)



- peak width at half-height ($w_{1/2}$)
  - typically less than 1 s for CE separated peaks
  - given in time units [s or min]

$$R_S = \frac{2(t_B - t_A)}{w_A + w_B}$$

- resolution (R_S, two consecutive peaks A and B)
- number of theoretical plates (N), i.e., the separation efficiency (individual peaks)

$$N = 5{,}545 \cdot \left(\frac{t_{migr}}{w_{1/2}} \right)^2$$

- peak area (A)
  - proportional to the sample concentration, used for quantitation purposes

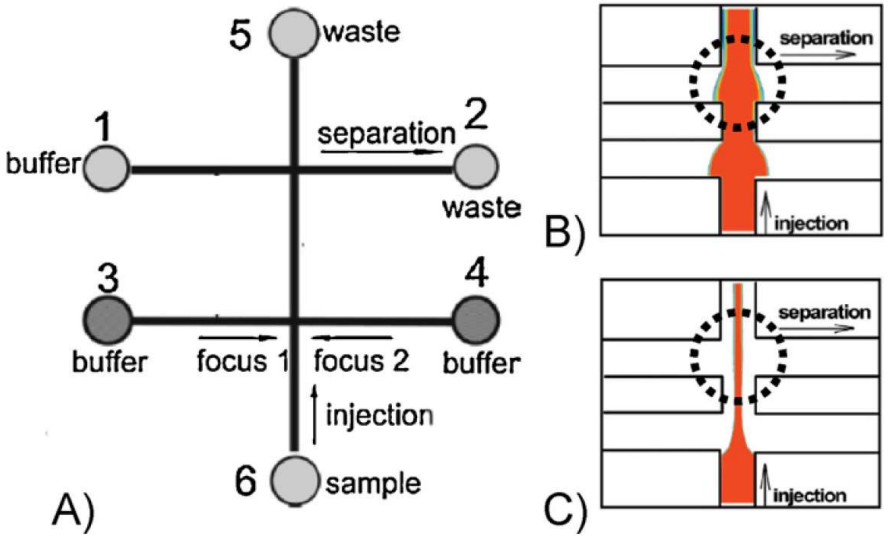
Impacts of microchip design and materials on separation performance

Injection vs. separation performance

Very narrow injected plug length is the key to better performance!

⇒ Less time (only few seconds!) required for separation of sample components
⇒ High resolution is achieved with shorter (~cm) separation lengths
⇒ Higher electric field strenghts can be applied over short separation channels

Example on how to narrow down the initial plug length even more:

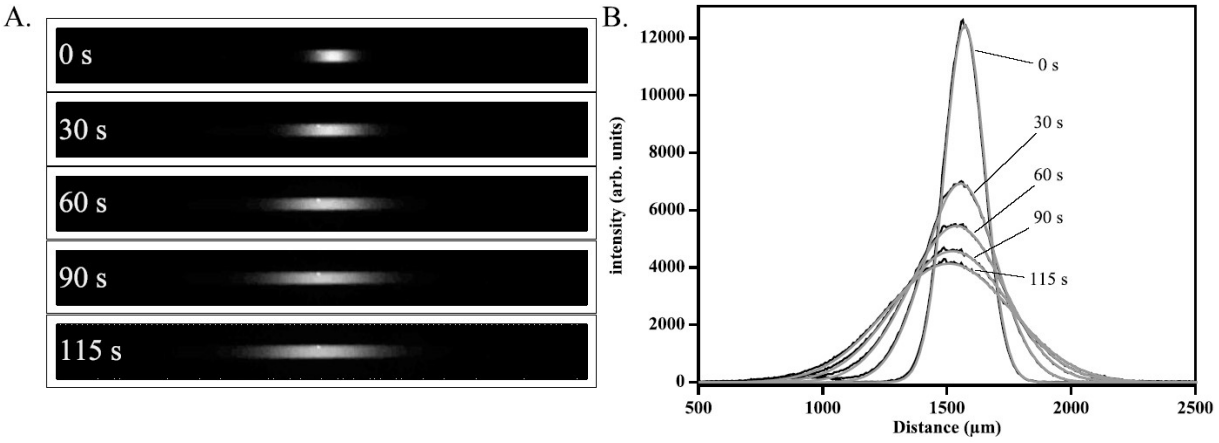


R.-J. Yang, et al., *J. Sep. Sci.* **2002**, 25, 996.

Peak width vs.
Dispersion and band broadening

- rule of thumb: the narrower peaks, the better the separation efficiency (*N*)

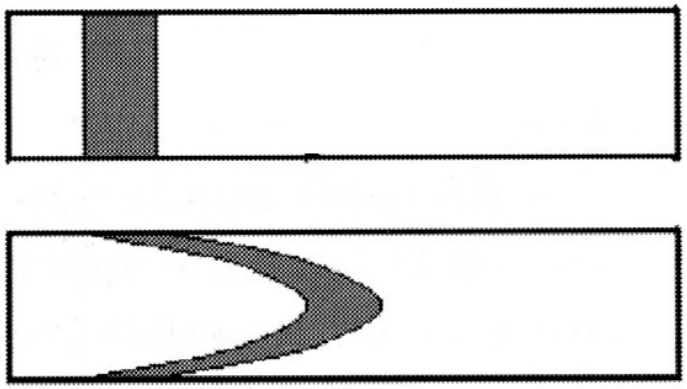
- major factors contributing to peak/band broadening
  1. **diffusion; accelerated by Joule heating → symmetrical effect**



Peak width vs. Dispersion and band broadening

- rule of thumb: the narrower peaks, the better the separation efficiency (*N*)

- major factors contributing to peak/band broadening
  1. diffusion; accelerated by Joule heating → symmetrical effect
  2. **pressure anomalies within separation channel**
     → peak fronting (positive pressure)
     → peak tailing (negative pressure)



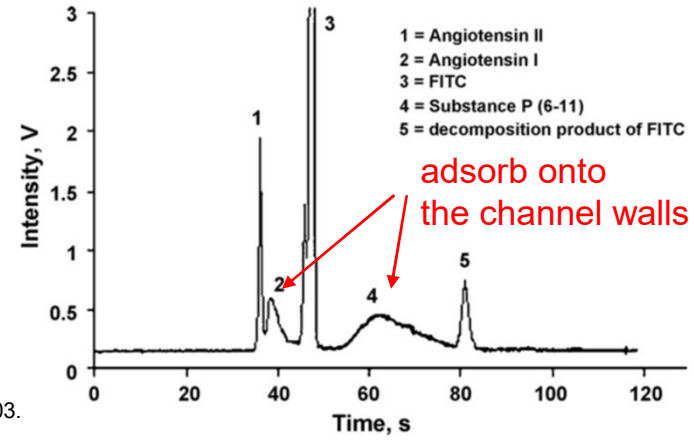
No pressure difference

Positive (back) pressure
→ Fronting peak

Peak width vs. Dispersion and band broadening

- rule of thumb: the narrower peaks, the better the separation efficiency (*N*)

- major factors contributing to peak/band broadening
  1. diffusion; accelerated by Joule heating → symmetrical effect
  2. pressure anomalies within separation channel
     → peak fronting (positive pressure)
     → peak tailing (negative pressure)
  3. **non-specific adsorption to surface → peak tailing**



Aura S et al., Sensors Act B 132, **2008**, 397-403.

Materials' effects

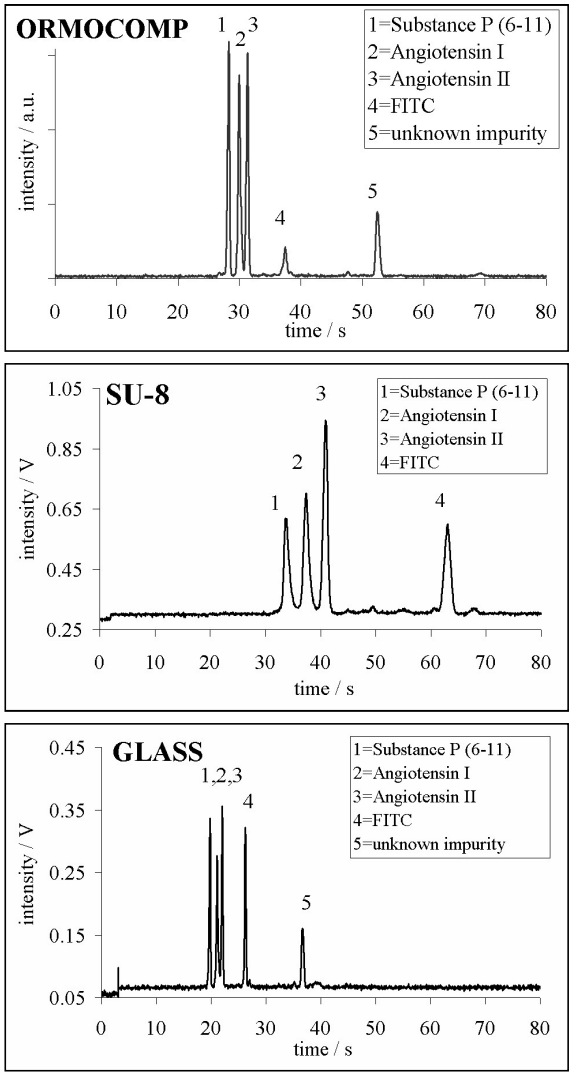
Separation rules in microchip CE are exactly the same as in capillaries, but fabrication materials/coatings contribute to performance via variation in surface charge (and nonspecific adsorption).

<u>Comparison of three different chip fabrication materials in microchip CE (identical conditions)</u>

- migration time repeatability 2-4% (RSD) all materials

- peak widths
  - ORMOCOMP 0.42-0.44 s
  - SU-8 1.04-1.42 s
  - glass 0.28-0.32 s

- number of theoretical plates
  - ORMOCOMP $6.6\text{-}8.0 \times 10^5$ /m
  - SU-8 $1.1\text{-}2.2 \times 10^5$ /m
  - glass $3.8\text{-}6.8 \times 10^5$ /m



Sikanen T et al., Anal. Chem. 79, **2007**, 6255.
Sikanen T et al., Anal. Chem. 82, **2010**, 3874.

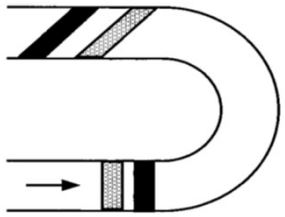
Impact of separation channel geometry (1)

- rule of thumb: the narrower peaks, the better the separation efficiency (*N*)

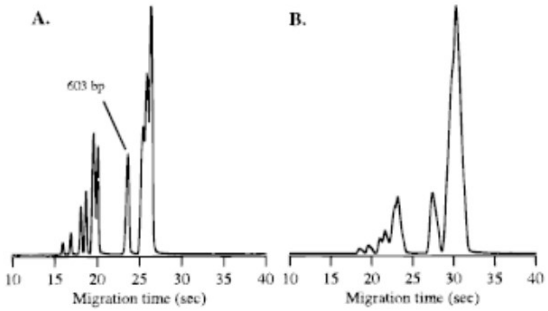
- major factors contributing to peak/band broadening
  1. diffusion; accelerated by Joule heating → symmetrical effect
  2. pressure anomalies within separation channel
     → peak fronting (positive pressure)
     → peak tailing (negative pressure)
  3. non-specific adsorption
     to surface → peak tailing
  4. **separation channel geometry**
     (e.g., meandering shape)

After channel turn

Before channel turn

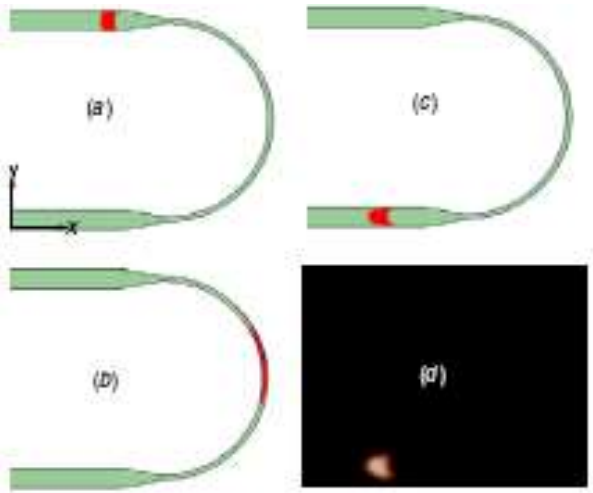


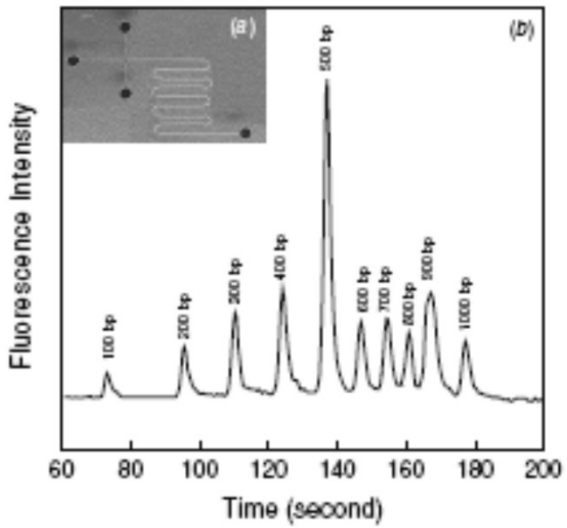
Before turn **After turn**



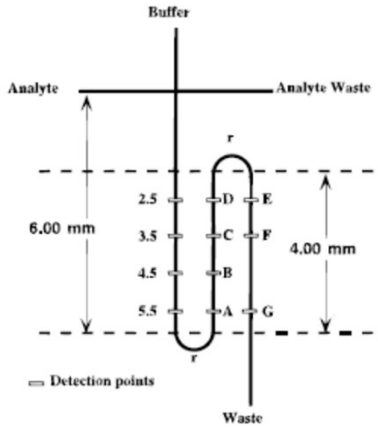
Impact of separation channel geometry (2)

- serpentine/meandering or spiral-shaped microchannels
  - Overall, save of a lot of space (and cost)
  - But are a source of band broadening
  - Common solution: compensation structures (e.g., tapering turns, see below)





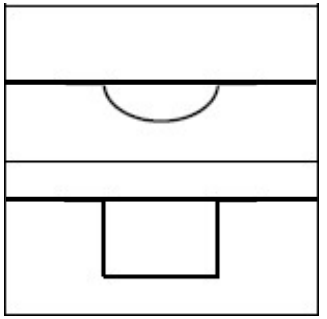
Tsai, C.H., et a., J. Micromech. Microeng. 2005, 15, 377–385.

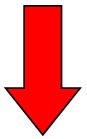


Culbertson, C.T., et al.,
Anal. Chem. 1998, 70, 3781-3789

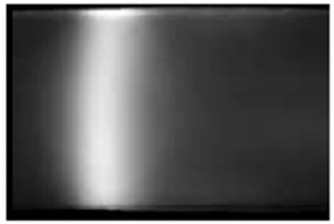
Impact of separation channel geometry (3)

- However, the microchannel cross-section layout (determined by the fabrication material and method) has no significant impact of peak width
  - glass/quartz: isotropic, i.e., ”semicircular”
  - polymers: almost always rectangular

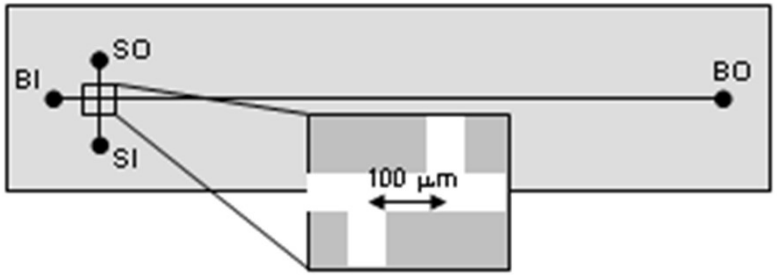




No significant effect on the flow profile

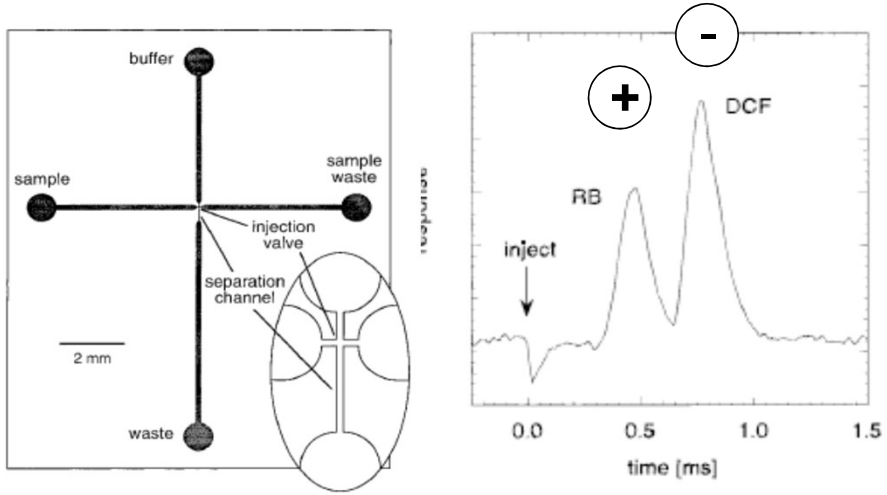


- Standard CE separation chip layout (top view)
  - straight separation channel (BI→BO, ~centimeters)
  - short intersecting channel (SI→SO) for injection
- Channel cross-section typically w=50 μm, h=20-50 μm



Pushing the Limits of Microchip-CE

- sub-millisecond separation of a binary sample on a glass chip
  - separation path length 200 μm
  - narrow separation channel dimensions w=26 μm, wider sample introduction channel w=200 μm
  - E = 53 kV/cm (input: 1 V per 6.1 V/cm)

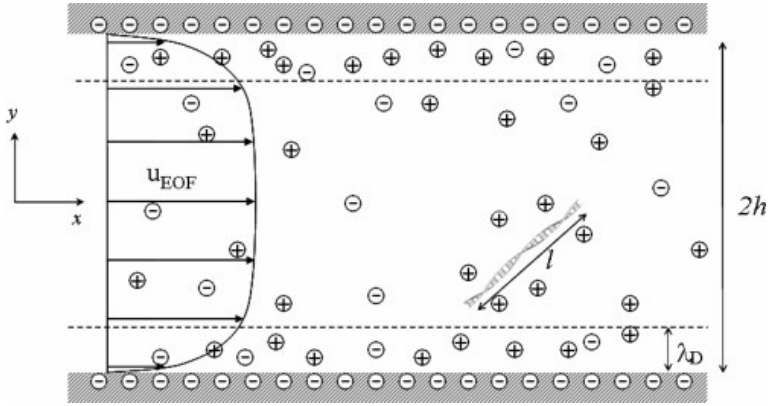


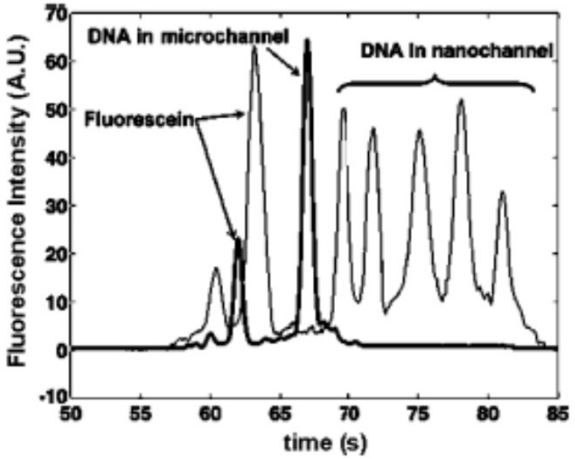
Jacobson, S.C., et al., Anal. Chem. 1998, 70, 3476-3480.

Physical Limits of CE on Chip - Nanochannels

- nanochannels (i.e., h < 1 μm)
  - EDL thickness/Debye length of the same order than the microchannel height
  - Analytes undergo transverse electromigration in addition to streamwise migration → steric structure (e.g. of DNA) also play a role
- Picture on the right:

  separation of DNA in nanochannels (h=100 nm) vs. microchannels (h=50 μm)





Pennathur, S., et al., Anal. Chem. 2007, 79, 8316-8322.

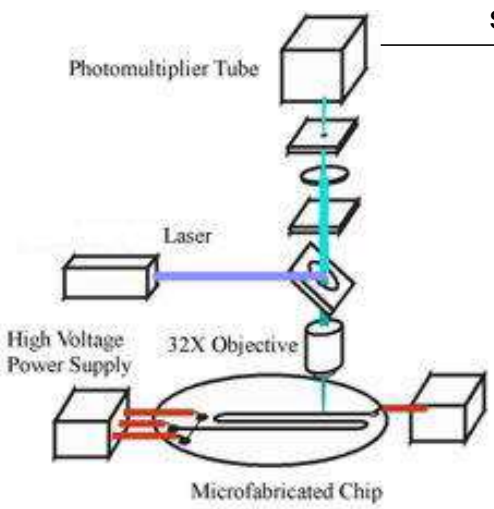
Detection possibilities:

Optical (most common)

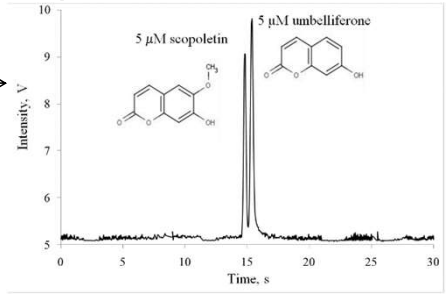
Electrochemical (easiest to miniaturize)

The most common setting

- detection setup typically comprises of a microchip placed on the sample stage of a (laser-induced) fluorescence microscope

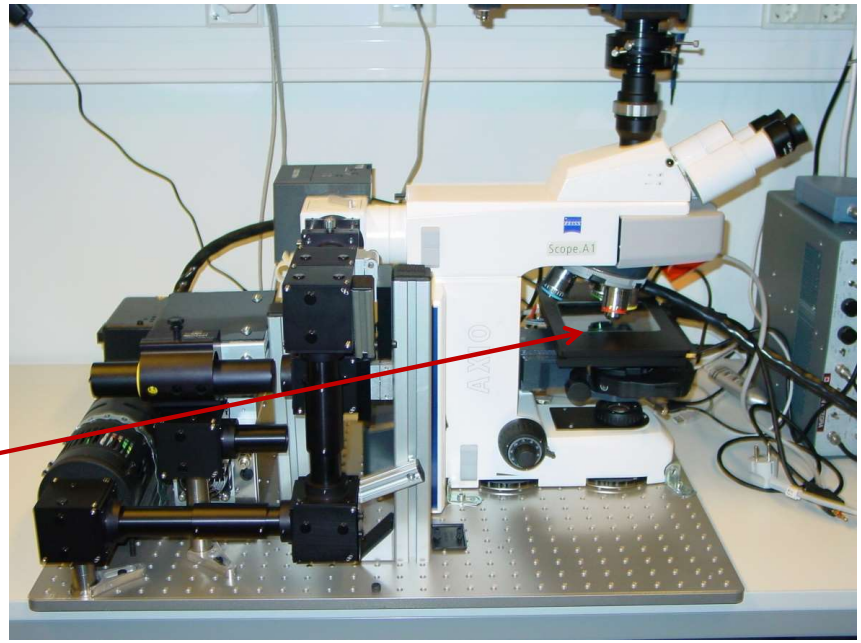


signal



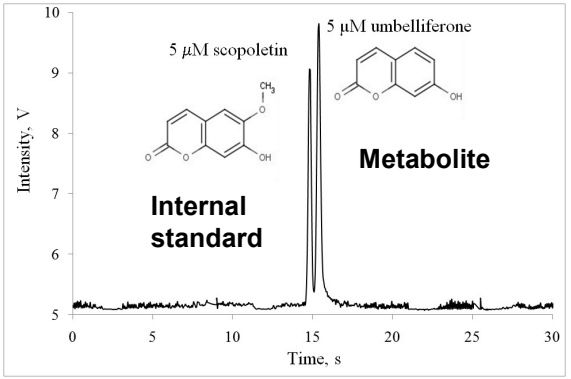
Sikanen T et al., Anal Chem 2010, 82, 3874.

Hutt LD et al., Anal. Chem. 1999, 71, 4000.

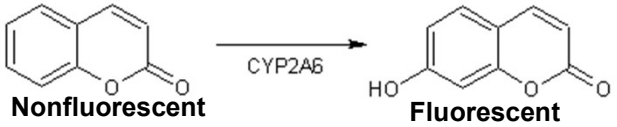


Fluorescence detection on-chip

Example: Coumarin 7-hydroxylation (a model reaction of CYP2A6 activity)

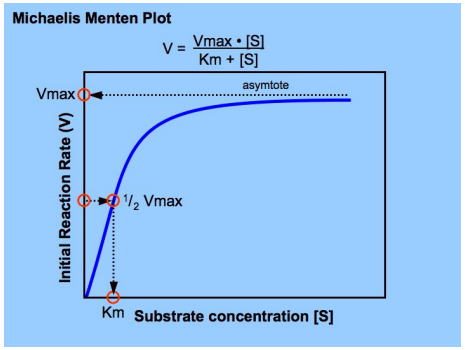


Sikanen T et al., Anal Chem 2010, 82, 3874.



Microchip electrophoresis method validation parameters:

- Quantitativity comparable to standard HPLC-UV
- Limit of detection 207 nM (~21×10^{-18} mol / 100 pL injected volume)
- Linearity R^2=0.9939 (between c=500 nM…2.5 µM) with 5 µM scopoletin as internal standard



	K_M [µM]	V_{max} [pmol/min/mg protein]
Microchip	6.0 ± 1.2	957 ± 40
In-house LC-UV	8.2 ± 3.2	1184 ± 143
Literature*	0.2-2.3	n/a

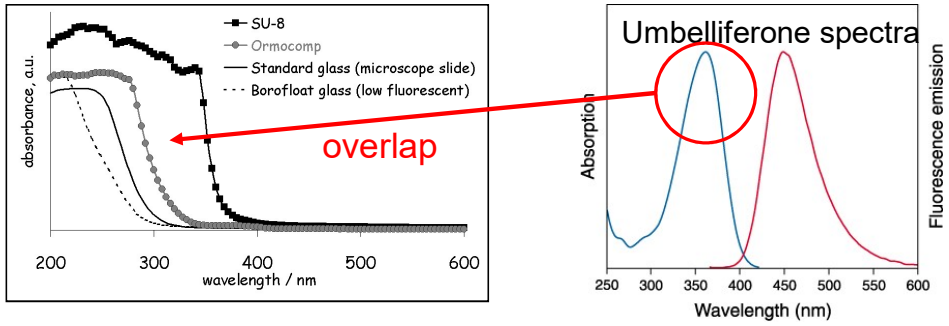
** Pelkonen O et al., Br. J. Clin. Pharmacol. 1985, 19, 59.*
Pearce R et al., Arch. Biochem. Biophys. 1992, 298, 211.
Draper AJ et al., Arch. Biochem. Biophys. 1997, 341, 47.

Detection Sensitivity vs. Miniaturization

- Lambert-Beer's law: $A = abc$

A	absorbance/signal intensity
a	absorptivity (specie-specific)
b	optical path length
c	sample concentration

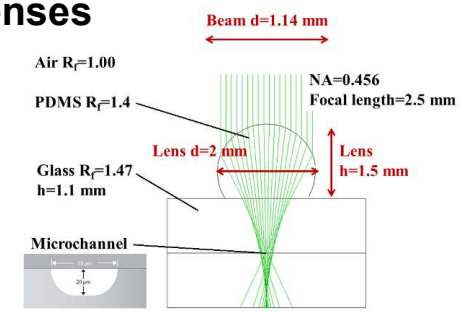
- Challenges related to optical detection sensitivity:
  - miniaturization reduces the optical path length (b)
    → lowers signal intensity → absorbance detection practically impossible
  - many polymers strongly absorb UV light
    → absorbance detection not possible
    → material autofluorescence increases noise in fluorescence detection

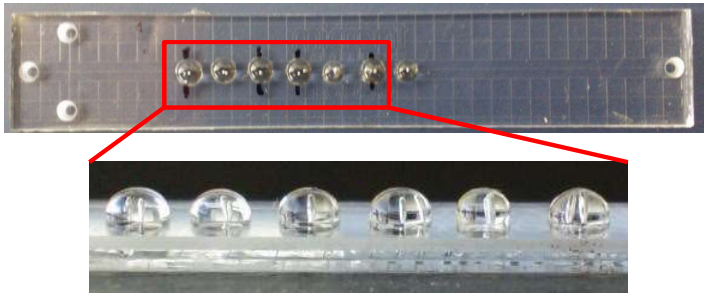


Some solutions to overcome the limited sensitivity of on-chip optical detection

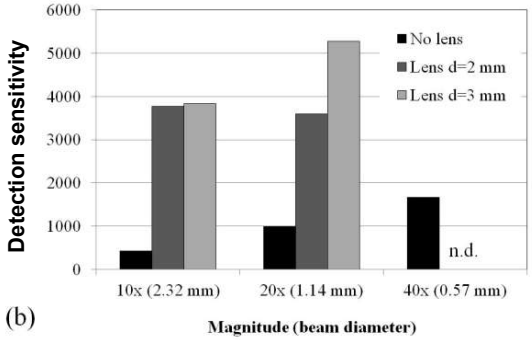
Microoptical elements for beam focusing (vertical microlenses) and reflection (micromirrors)

Microlenses



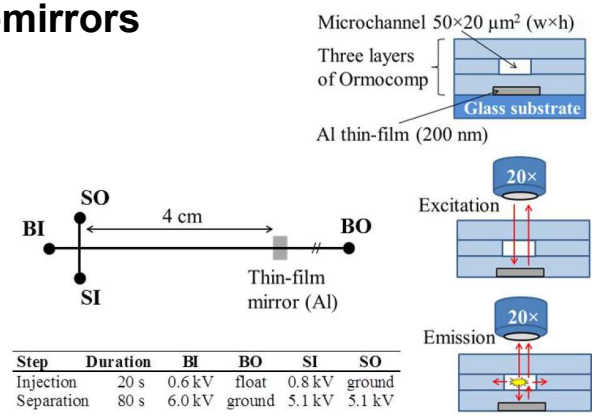


Nordberg, M.E., et al., MicroTAS 2014, San Antonio, TX.

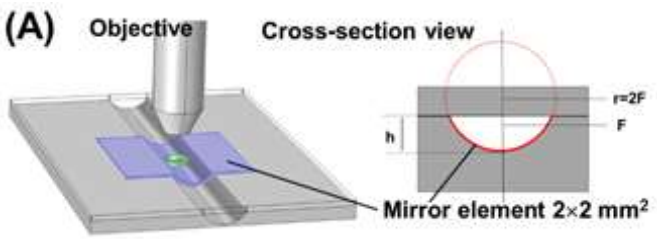


(b)

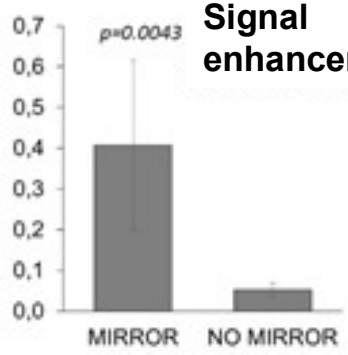
Micromirrors



Step	Duration	BI	BO	SI	SO
Injection	20 s	0.6 kV	float	0.8 kV	ground
Separation	80 s	6.0 kV	ground	5.1 kV	5.1 kV



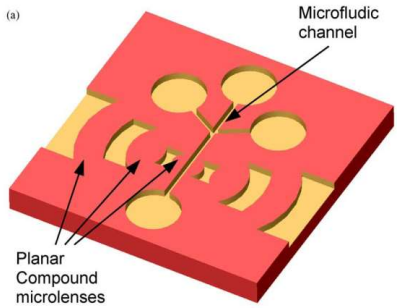
Bonabi, A., et al., Biomicrofluid. 11, 2017, 034118

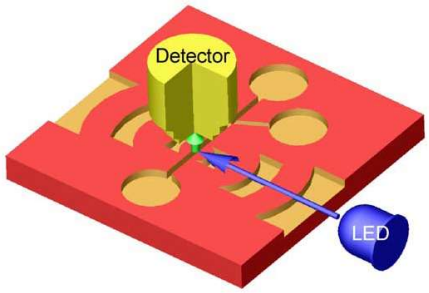


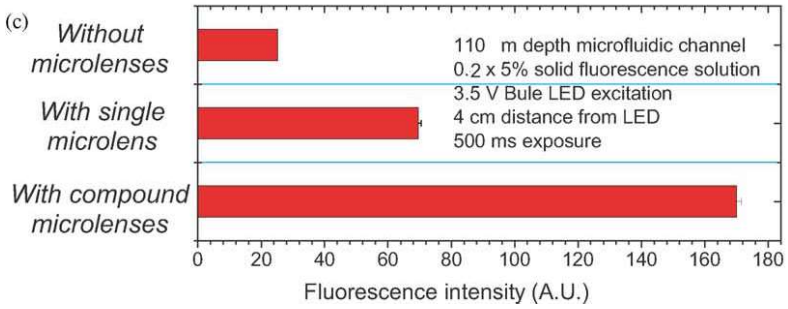
Signal enhancement

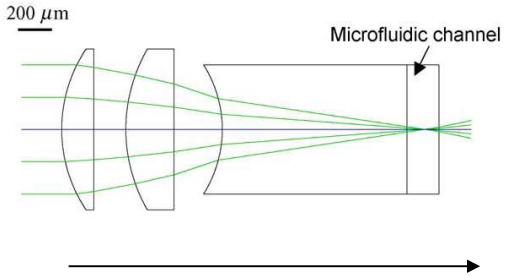
Some solutions to overcome the limited sensitivity of on-chip optical detection (2)

- ANOTHER SOLUTION: integration of horizontal microlenses for beam focusing
  - Designed for coupling to external, miniaturized light sources
  - Allows for multiple compound lens arrays → more efficient beam focusing (but only in 2D!)









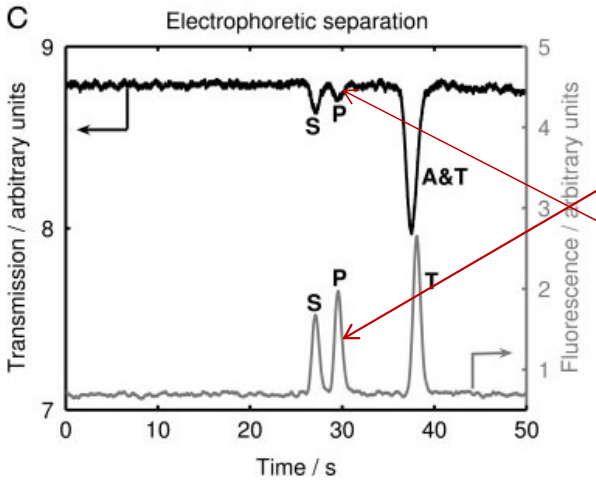
Direction of the beam

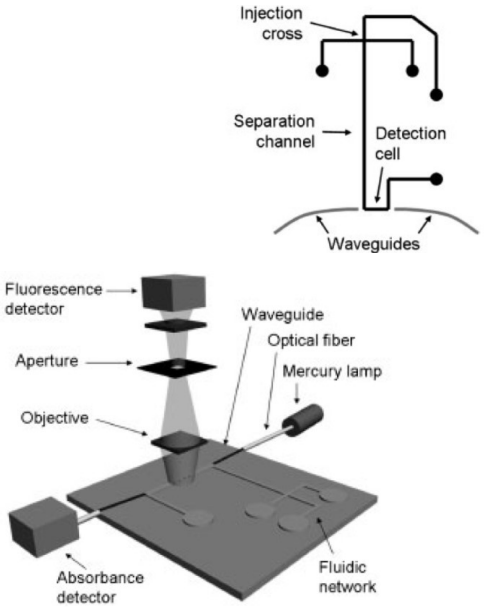
J. Seo and L.P. Lee, Sens. Actuators B 2004, 99, 615-622.

Some solutions to overcome the limited sensitivity of on-chip optical detection (3)

- YET ANOTHER SOLUTION: increasing the optical path length of horizontal beam traveling along the microchannel (detection cell)
  - Even UV absorbance detection can be achieved
  - But: may significantly limit the resolving power (R_s) of analytes that migrate close to each other and thus enter the detection cell at the same time

Compound	LOD (μM)		
	Absorbance	Native UV fluorescence	
	This work	This work	Previously reported[a)]
Serotonin	14	4	0.113–59
Tryptophan	14	2	0.025–63
Propranolol	31	3	0.081–1.4
Acetaminophen	3	>100	–

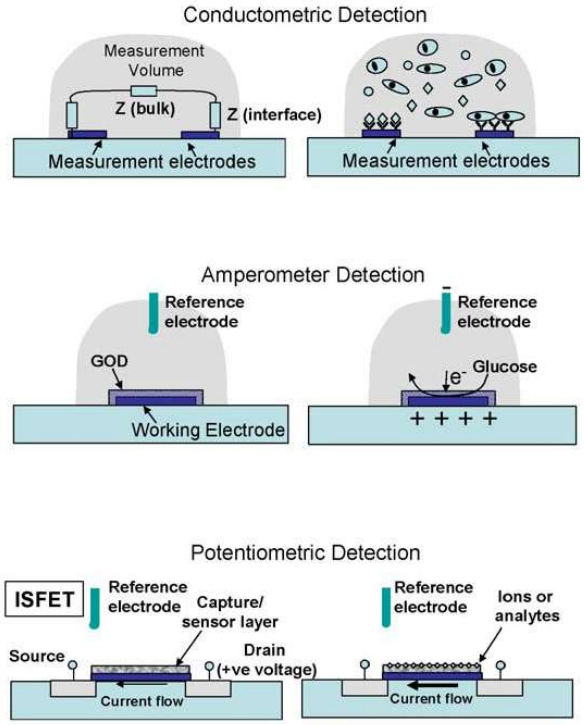




P.D. Ohlsson et al., Electrophoresis 2009, 30, 4172–4178.

Electrochemical Detection – Three Modes

- More universal than fluorescence detection (though only feasible for electroactive compounds)
- Detector elements (electrodes) can be patterned by thin-film metallization
- Benefits from detector miniaturization (S/N ↑) unlike optical detection



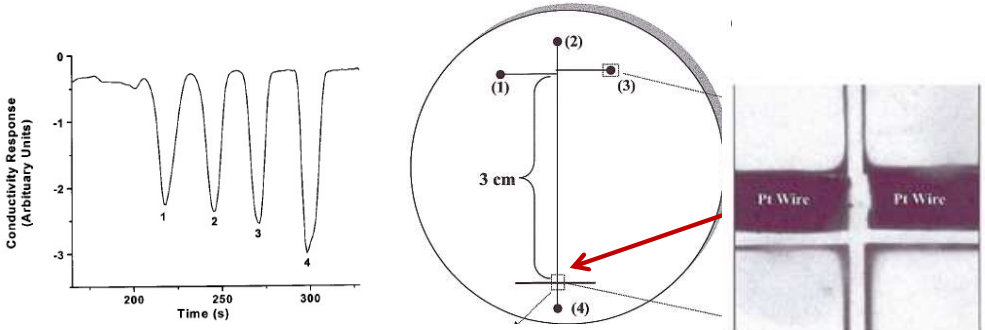
Operation principles:

- Measures ion concentration between electrodes
- Working electrode measures electrons generated by redox reaction
- Potential measured against a reference electrode

Conductivity Detection on Chip

(1) Electrodes <u>in contact</u> with liquid

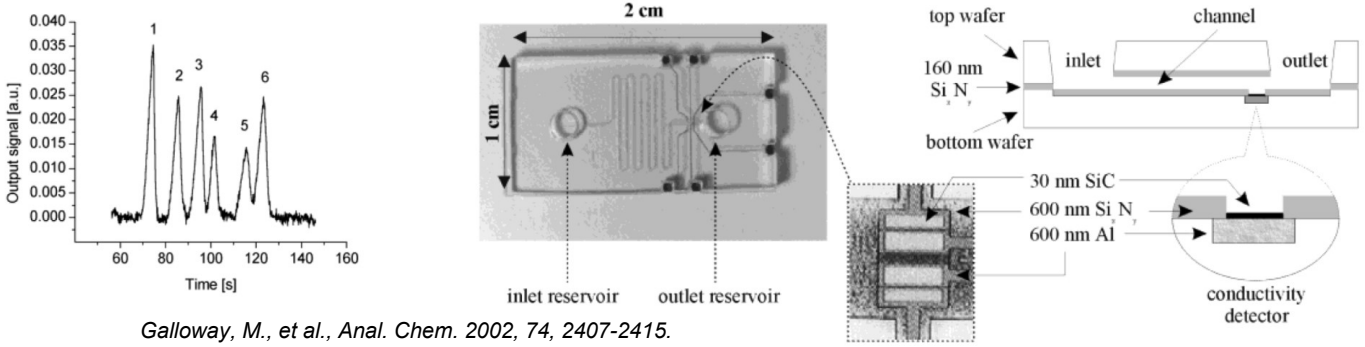
- More sensitive to corrosion → only noble metals can be used (Au, Ag, Pt)
- Bonding (of cover layer) over metals may be problematic



Galloway, M., et al., Anal. Chem. 2002, 74, 2407-2415.

(2) <u>Contactless</u> = electrodes not in contact with liquid

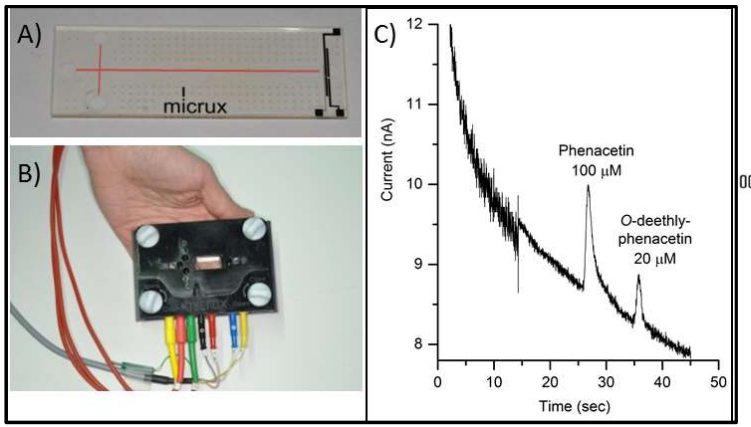
- Can be embedded on chip → bonding & corrosion problems avoided → more metals available

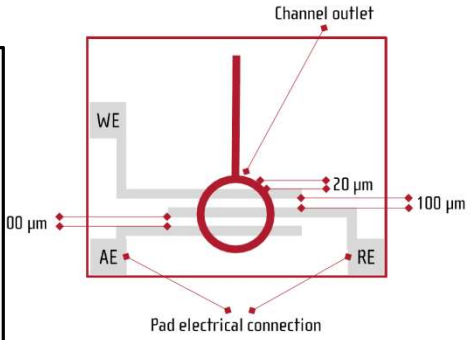


Galloway, M., et al., Anal. Chem. 2002, 74, 2407-2415.

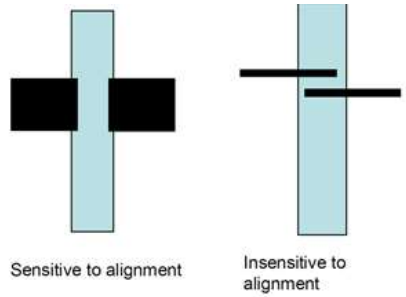
Amperometric Detection on Chip

- Electrodes always in contact with the sample solution
  (oxidation/reduction reactions occur on the surface of the electrodes)
- Design considerations:
  - Electrodes are typically placed at the outlet to avoid blocking of the channel with gas (bubbles) produced at the electrodes (redox reactions)
  - Sensing electrodes have to be isolated from HV electrodes (needed EOF actuation)
  - Alignment vs. repeatability





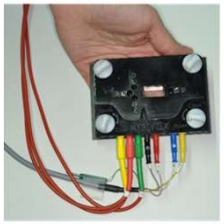
http://www.micruxfluidic.com/



» **WE:** working electrode
» **RE:** reference electrode
» **AE:** auxiliary electrode

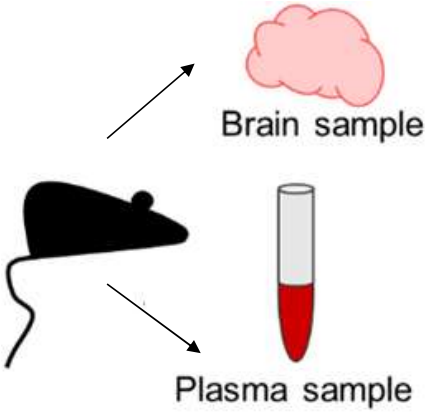
Ollikainen, E., et al., Proc. MicroTAS 2015, Gyengju, The Republic of Korea, 2015, pp. 2005-2007.

Determination of drug concentrations in mouse plasma and brain



Commercial microchip electrophoresis platform with bipotentiostat
→ Complementary chemical data for a behavioral pharmacological study

Mouse brain homogenate (~mL)

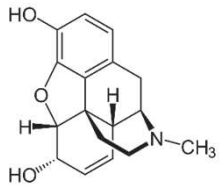


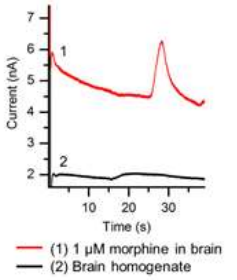
Liquid-liquid extraction

Mouse plasma (~μL)

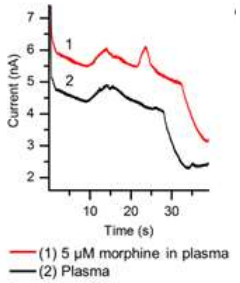
Purification & 16-fold enrichment

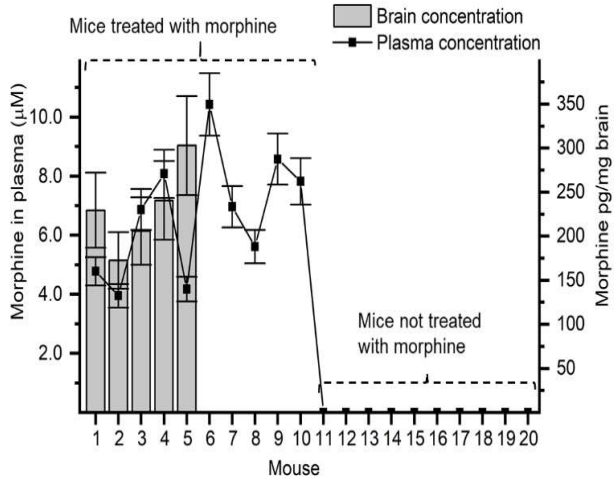
Target compounds: Morphine





Purification & 2-fold enrichment





Ollikainen, E., et al., Sci Rep. 2019, 9, 3311 (9 pp).

Mass spectrometry

(a rarer alternative to very selective detection)

Mass spectrometry

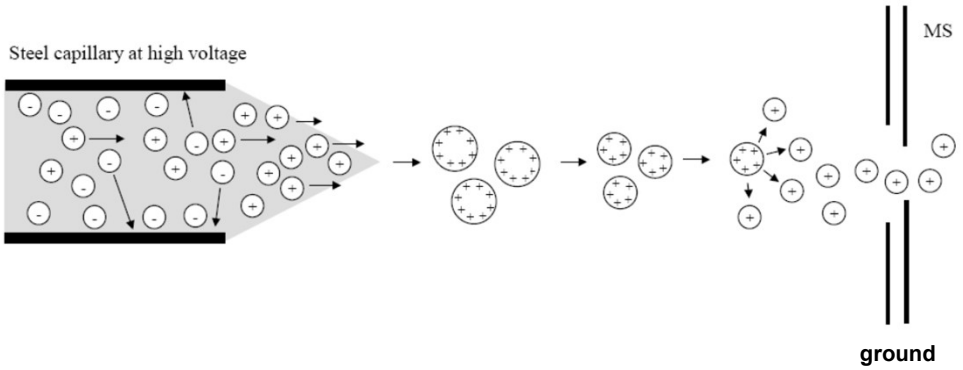
- Sample solution (liquid) needs to be ionized prior to introduction into a mass spectrometer

A conventional mass spectrometer comprises of

- ***An ion source → Easier to miniaturize***
  - Electrospray ionization (by far the most common ionization mode, compatible with CE)
  - Chemical ionization, photoionization etc. also possible

- ***A mass analyzer (of gas phase ions) → Very niche field of research when it comes to miniaturization***
  - Expensive infrastructure

Electrospray ionization (ESI) principle

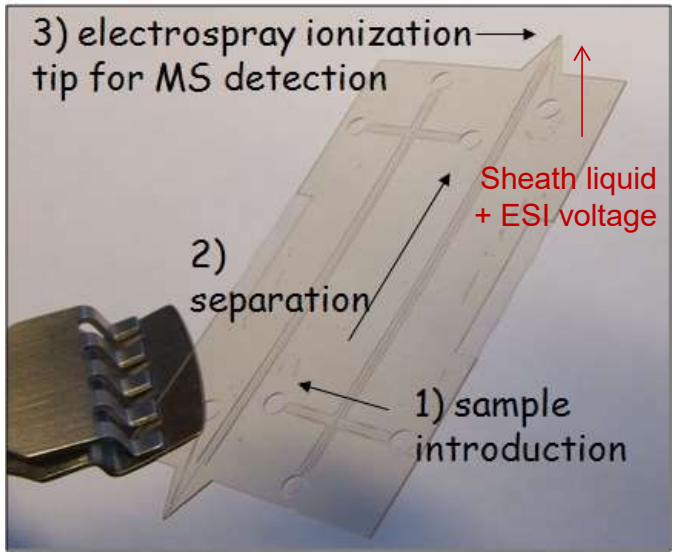


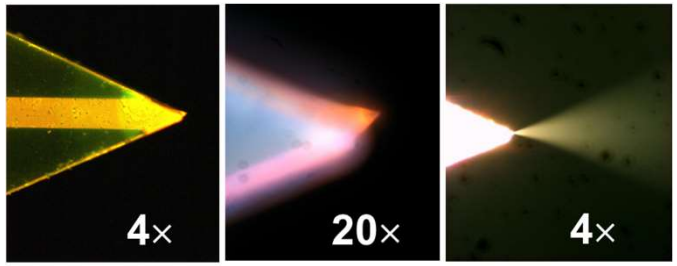
https://en.wikipedia.org/wiki/Electrospray_ionization

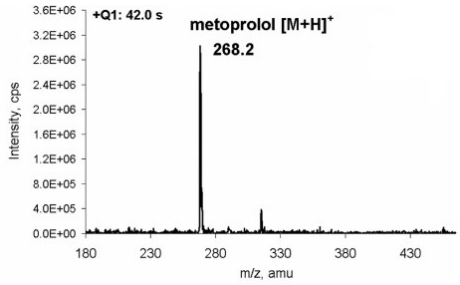
Mass spectrometric detection ”on chip”

The main challenge in miniaturization of an electrospray ion source:

- Stability of the ionization process → requires fabrication of a sharp emitter tip (demanding for most microfabrication methods and materials)







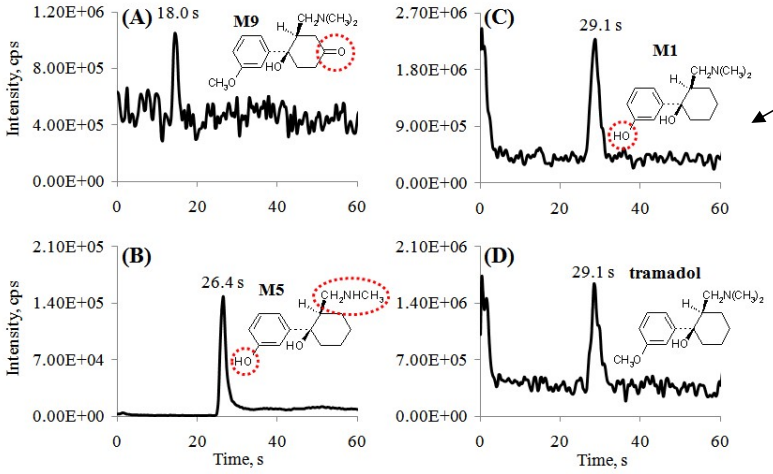
In the ion source:
Sample molecules are transferred from liquid to gas phase via coulombic repulsion under high electric field (ion emission and evaporation of the solvent)

In the mass analyzer:
The gas phase molecules are detected based on their mass-to-charge ratio (m/z)

Sikanen T et al., Anal. Chem. ***2007****, 79, 9135.*

Application example: CE chip with a fully integrated on-chip emitter for electrospray ionization

Separation of drug (tramadol) metabolites in urine by microchip electrophoresis-electrospray ionization MS

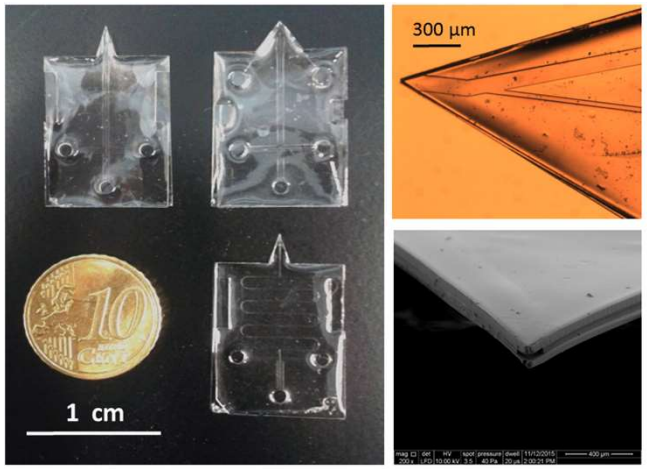


	Metabolite	*m/z*	MS mode*	pK_a		logD	
				Amine	Phenol	pH 11	pH 3
(A)	M9	278.6	EIE	9.28	-	1.26	-2.23
(B)	M5	236.1→44.2	SRM	10.02	9.22	0.34	-1.32
(C)	M1	250.5	EIE	8.97	9.62	0.74	-1.20
(D)	tramadol	264.3	EIE	9.23	-	2.44	-1.05

*EIE=extracted ion electropherogram, SRM=selected reaction monitoring

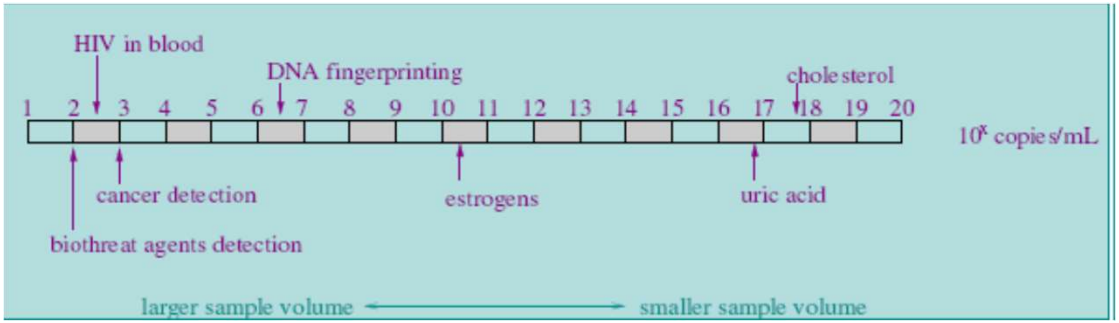
Nordman et al., J. Chrom. A 2011, 1218, 739-745.

Each m/z ion current can be extracted from the total ion current to quantitate the peak area (signal intensity)



Tähkä, S., et al., J. Chromatogr. A, 1496, 2017, 150-156.

Choice of the Detection Method



- The required and obtained sensitivity is largely dependent on the target application
  - All analytical methods are specifically tailored for each application, no method covers all concentrations and/or all target compounds
  - Sample pretreatment before analysis/detection is mandatory for low concentrations
- Spectroscopic methods (absorbance, fluorescence) → Detect atomistic properties
  - Choromophore/fluorophore (in the molecular structure) required for optical detection
  - Many organic solvents and microchip materials also absorb UV light
- Electrochemical methods → Detect bulk properties (conductivity, current)
  - Electroactive functional group (in the molecular structure) is required
  - Many backgroud electrolyte ions may also oxidize/reduce
- Mass spectrometry → Detects m/z, by far most selective and thus sensitive
  - Fairly expensive instrumentation (mass analyzers) required
  - Miniaturization of the ion source typically requires specialty fabrication protocols

Back to separation systems

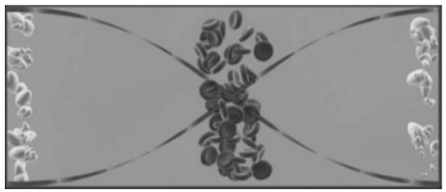
Overview of the main separation techniques

Electrophoresis	Chromatography
ACTUATION PRINCIPLE:	
▪ electroosmotic flow	▪ pressure driven flow
SEPARATION PRINCIPLE:	
▪ electromigration based on size and charge or pI	▪ distribution/adsorption to solid support
SEPARATION MODES:	
▪ capillary (free zone) electrophoresis (CZE)	▪ liquid (LC) or gas (GC) chromatography
▪ isoelectric focusing (IEF)	▪ capillary electro-chromatography (CEC)
▪ micellar electrokinetic chromatography (MEKC)	▪ **lab-on-cd** approach → actuation by centrifugal forces
▪ gel electrophoresis (i.e. PAGE)	

At focus so far

Separation of cells and particles (excluded)

- **acoustophoresis**
  - standing high frequency sound waves

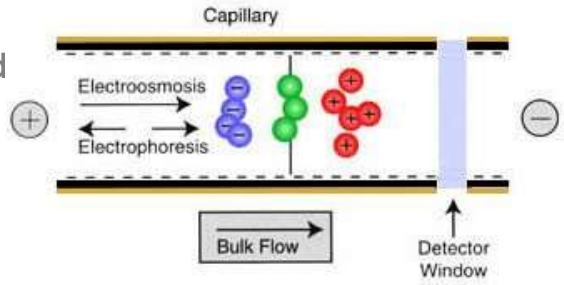


Laurell, T., et al.,
Chem. Soc. Rev., 2007, 36, 492–506.

- **magnetophoresis** and other field-flow fractination (FFF) techniques
  - -field perpendicular to flow (gravitational, thermal…)
  - -separation mainly based on differences in diffusion and/or Brownian motion

Other electrophoretic separation modes

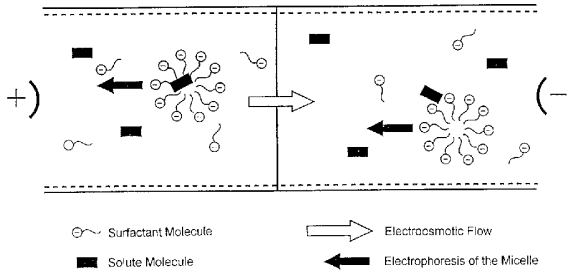
- **Capillary (Zone) Electrophoresis (CZE, CE) = most used**
  - separation based on size and charge state
  - control of electroosmotic flow (EOF) by surface charge



***Other modes of separation*:**

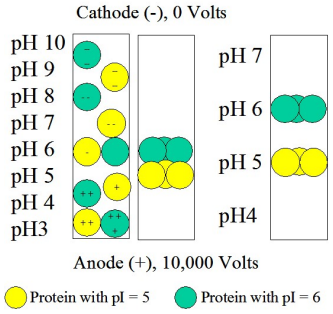
- **Micellar/Liposome Electrokinetic Chromatography (MEKC, LEKC)**
  - inclusion of surfactants (hydrophobic-hydrophilic moieties, e.g., SDS, Tween, Triton X…)
  - critical micelle concentration (CMC)

    → MEKC or just surface modification?

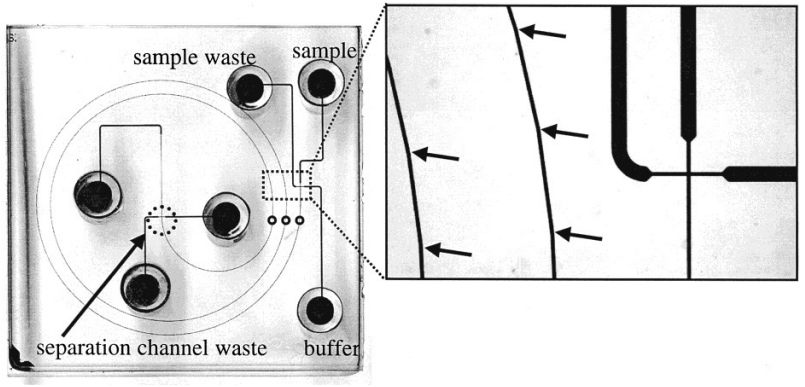


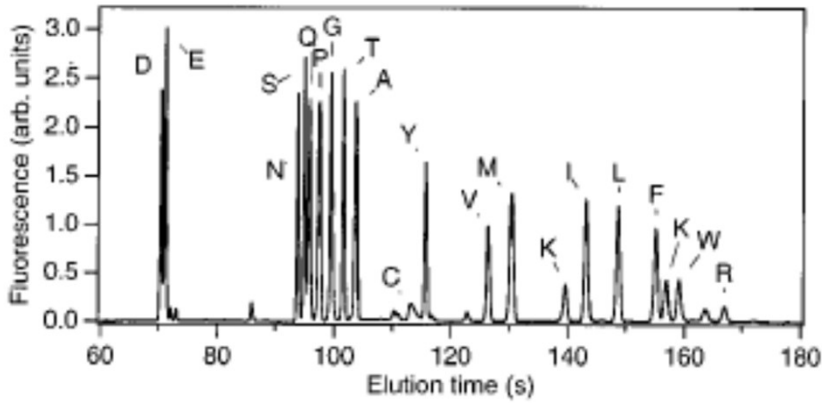
- **Capillary IsoElectric Focusing (CIEF, cIEF or simply IEF)**
  - elimination of EOF and inclusion of carrier ampholytes (zwitterions)

    → formation of pH gradient → separation based on pI
  - in capillaries (cIEF) or gel (e.g., polyacrylamide gel (PA)-GE)
- **Gel Electrophoresis (GE)**
  - typically based on IEF in gel, sometimes on size exclusion



Example of micellar electrokinetic chromatography on chip





Culbertson, C.T., et al., Anal. Chem. 2000, 72, 5814-5819.

- separation of 19 amino acids in less than 3 minutes !
  - up to 10^6 theoretical plates
  - glass chip with 25-cm-long separation channel (spiral)
  - E > 1000 V/ cm
  - MEKC conditions:
    10 mM sodium tetraborate,
    50 mM SDS, 10% 2-propanol
  - amino acids labeled with tetramethylrhodamine

About terminology

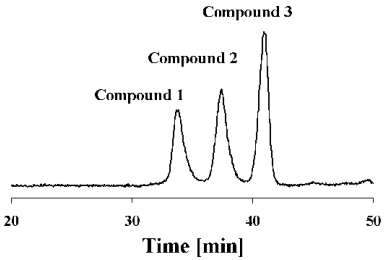
- Microchip electrophoresis
  - Mainly refers to capillary (zone) electrophoresis on chip
  - Common abbreviation ’MCE’ = microchip capillary electrophoresis
  - Sometimes abbreviated ’microchip-CE’ or simply ’ME’
  - May also include MEKC, but if so, it is typically separately emphasized
- If any other electrokinetic separation mode (than MCE) is used, it is typically indicated clearly (e.g., CIEF on chip)

Chromatographic separations on chip

Chromatographic separation techniques

- Separation principle:
  - Separation compartment (e.g., microchannel) is packed with stationary phase
  - Mobile phase = effluent is pumped (pressure-driven flow!) through the stationary phase
  - Sample components (dissolved in the mobile phase) interact with the stationary phase
    - components with least interactions pass the separation channel fastest

      → appear first at the detector (=compound 1); and vice ver
  - Overall separation efficiency (resolution) increases with increasing amount of interactions (surface area)

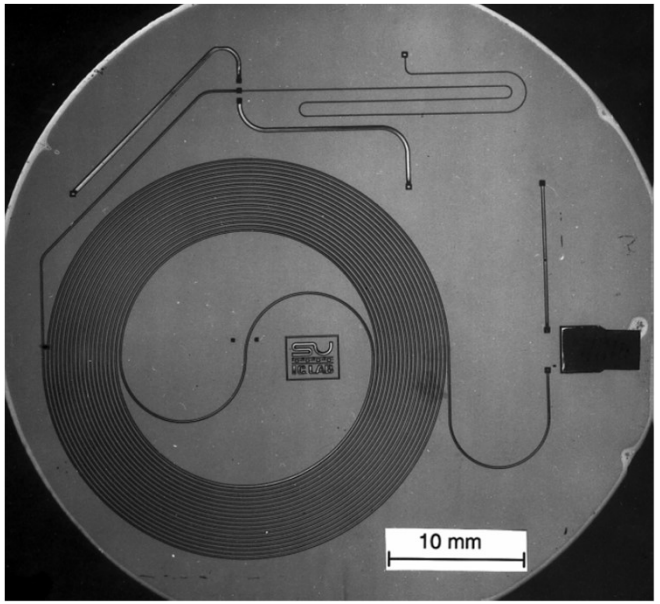


- Chromatographic techniques can be roughly divided in two
  - Gas chromatography (GC): mobile phase = gas (He); stationary phase = liquid or solid
    - separation channel walls are typically COATED with the stationary phase material
  - Liquid chromatography (LC): mobile phase = liquid; stationary phase = solid
    - separation channel is PACKED with the porous stationary phase material

      (microparticles 2-5 μm or porous monolithic material)

Gas chromatography on chip

- first ever on-chip separation
  - published already in the late 1970's !
  - a miniaturized gas chromatograph
    - sample injection system
    - 1.5 m separation column
    - a thermal conductivity detector
  - the chip was fabricated by standard photolithography and wet-etching



  - the separation channel coated with liquid stationary phase
  - helium as carrier gas

→ separation of gaseous hydrocarbon mixtures in less than 10 seconds !

Terry, S. C.; Angell, J. B. Des. Biomed. Appl. Solid State Chem. Sens., Workshop, Meeting date 1977, 1978, pp. 207-218

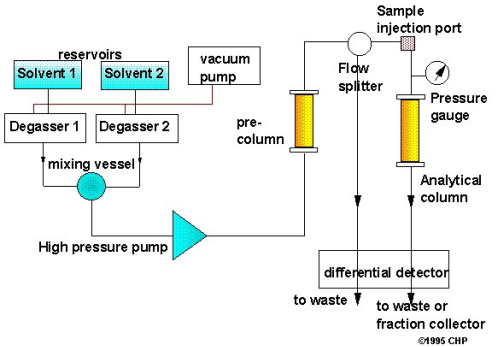
Liquid Chromatography (LC)

KEY COMPONENTS OF THE CONVENTIONAL SETTING

- Reversed phase LC – the standard case (more common than GC)
  - the stationary solid phase is typically C18 (or C8)
  - the mobile phase is typically a mixture of methanol-water or acetonitrile-water
  - compounds pass the separation channel in order of increasing hydrophobicity

$$\|\text{–}CH_2\text{–}(CH_2)_{16}\text{–}CH_3$$

- High pressure pumps are needed to provide sufficient liquid flow (mobile phase)
  - typically P~200-400 psi
  - typically two pumps are used – one for aqueous and one for organic solvent – in order to increase the amount of organic solvent in the mobile phase (e.g., methanol-water gradient from 10:90 to 90:10, vol/vol-%)



Miniaturization of LC instrumentation

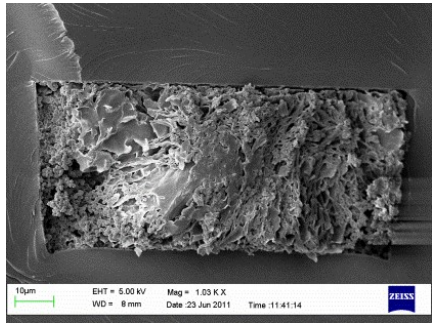
- Almost always combined with mass spectrometric detector
- Much less common than miniaturized electrophoretic instrumentation,

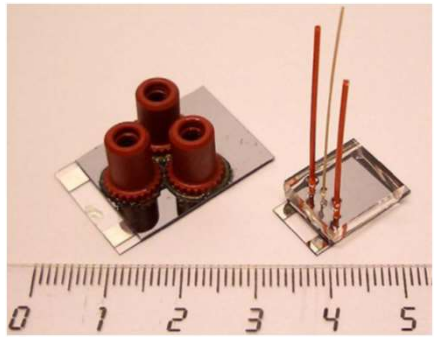
  because miniaturization of LC meets up with certain challenges

1. **How low can you go?**
   - chromatographic resolving power increases with increasing amount of interactions:
     → decreasing column length on a microchip
     → decreasing surface area → less interactions

2. **Immobilization of <u>homogenous</u>, stationary phase**
   - linkers (solid phase supports) required on walls
   - Corners are problematic → voids (dead volume, leakage)

3. **Application of pressure driven flow**
   - pressure-tight macro-to-micro interfacing with external pumps required
   - or alternatively, on-chip micropumps and mixers need to be incorporated
   - polymer (and glass) bonding cannot withstand high pressures

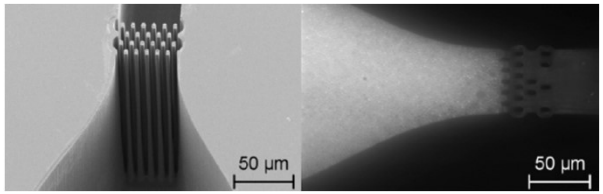




Immobilization of the stationary (solid) phase

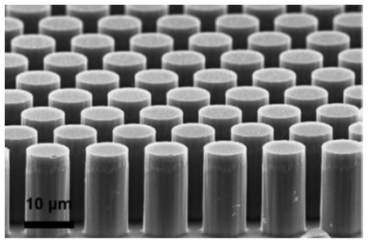
Haapala, M., et al., Anal. Chim. Acta 2010, 662, 163–169.

- **Alternative solid phase support structures:**
  - *MICROPARTICLES*
    - silica-based beads, polymer-based beads
    - coated with stationary phase material
    - bead diameter ~2-5 μm, pore size ~10-30 nm
    - pumped in as a slurry and maintained using micropillar frits as mechanical barriers

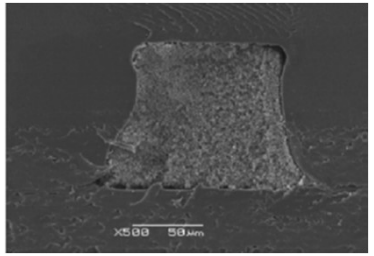


  - *MICROFABRICATED (SILICON) PILLARS*
    - coated with stationary phase material (post-fabrication)
    - microfabricated alternative to microparticles, but surface area typically lower than that of microparticles

Sainiemi, L., et al., Sens. Actuators B 2008, 132, 380-387.



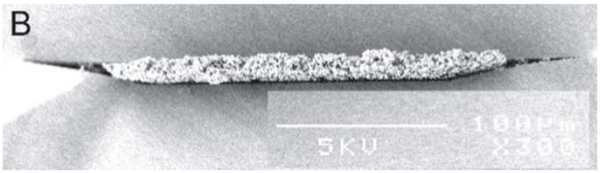
  - *MONOLITHS*
    - usually polymer-based, also silica-based
    - monomers + initiator → functional polymer monoliths
    - microchannel filled with monomer solution
      - UV-curing → UV transparent substrates needed
      - curing by heat → substrates with high T_g needed
    - porogens added to the monomer mixture for pore formation
      - large pores (>50-100 nm) enabling through-flow
      - small pores (<2 nm) for retention



Liu, J., et al., Int. J. Mass Spectrom. 2007, 259, 65–72.

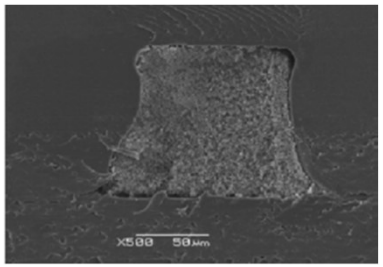
Impact of microchannel geometry on separation performance

- Microchannel cross-section layout
  - Mainly affects the uniformity of solid phase packing
  - If packing fails (e.g., corners) → leakage → band broadening/dispersion

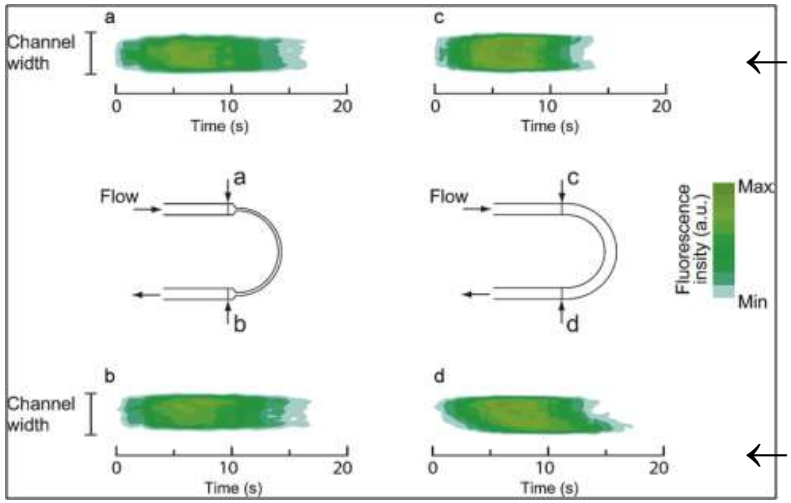


Ishida, A., et al., J. Chromatogr. A 2006, 1132, 90–98.

- Serpentine/meandering microchannels
  - Save of space (and cost)
  - Turns = source of band broadening/distortion (similar to microchip CE)
    → curve optimization through tapering



Liu, J., et al., Int. J. Mass Spectrom. 2007, 259, 65–72.



← BEFORE TURN

← AFTER TURN

Shape of the sample band in tapered and normal turn (as illustrated by fluorescence intensity distribution).

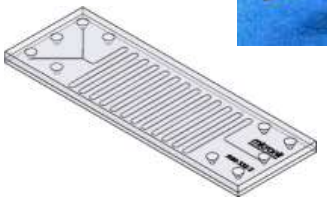
Ishida, A., et al., J. Chromatogr. A 2006, 1132, 90–98.

Application of pressure driven flow

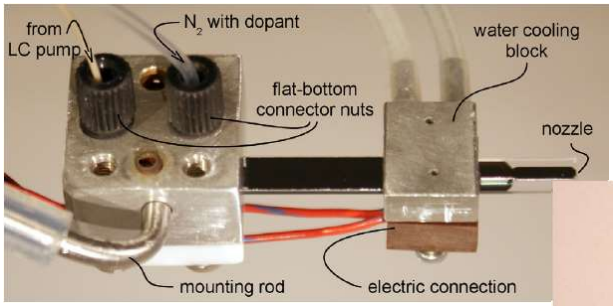
On-Chip vs. Off-Chip Pumping (and Mixing) – the Two Options

OFF-CHIP APPROACH

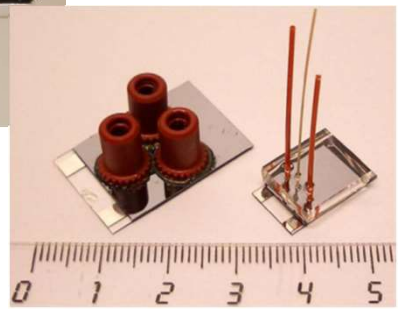
- Chip interfaced with macroscale pumps
  - Most commonly used



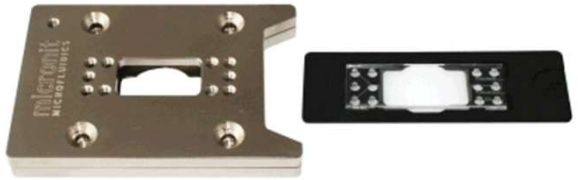
- Pressure-tight macro-to-micro interfacing needed
  - both commercial and custom-made solutions
  - a lot of manual work often required
    (e.g., gluing of nanoports etc. for capillary connections)



Haapala, M., et al., Anal. Chim. Acta 2010, 662, 163–169.



Saarela, V., et al., Sens. Actuators B 2006, 114, 552–557.



Fluidic Connect Kit from Micronit Microfluidics BV

Application of pressure driven flow

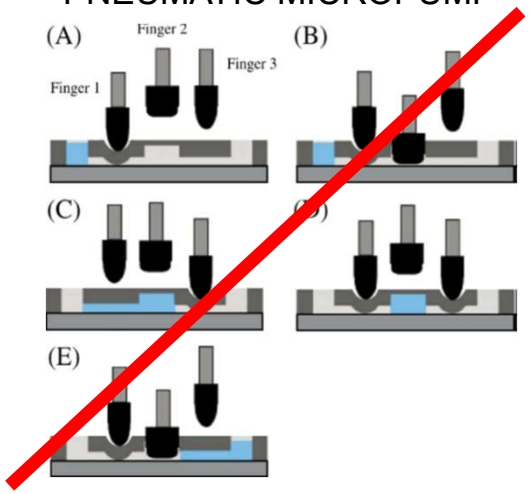
ON-CHIP APPROACH

- several possibilities for miniaturization and integration of micropumps on chip

  - mechanically controlled micropumps, e.g., piezoelectric, pneumatic, thermopneumatic…
    - None feasible for LC/GC

  - non-mechanically controlled micropumps
    - **<u>electrokinetic and electrochemical</u>**
      → high pressure tolerance
      → most common in on-chip chromatography
    - also others: magnetohydrodynamic, surface tension/capillary action, ferrofluidic, acoustic wave…

PNEUMATIC MICROPUMP

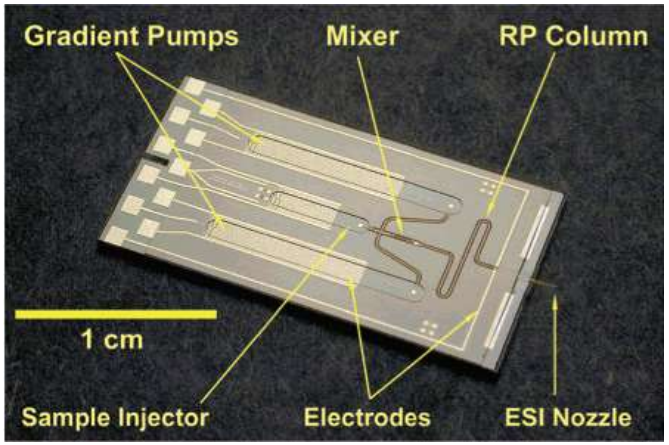


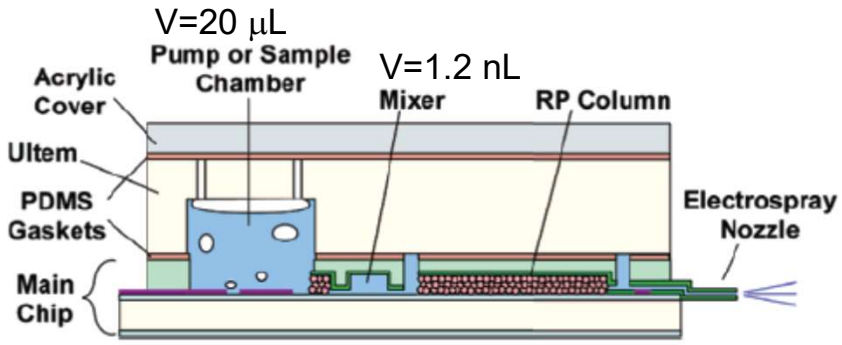
Examples of integrated on-chip pumps: Electrochemical approach

1. Galvanostatic control

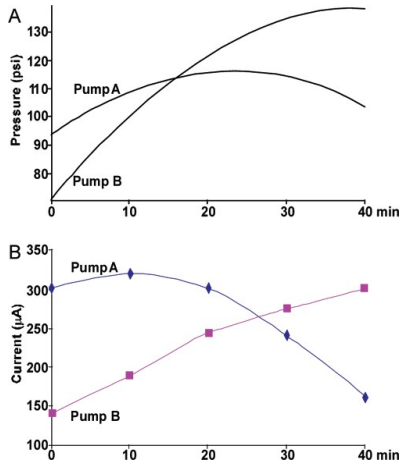
- Electrical current (0-400 μA) is ”converted into gas”
  → electrolysis of water produces gas (H_2)
  → gas replaces liquid in the solvent chamber

- Pumping pressure ~100-200 psi (7-14 bar)
- Liquid flow rates up to 120 nL/min
- Total power consumption ~2 mW





Xie, J., et al., Anal. Chem. 2005, 77, 6947-6953.



Examples of integrated on-chip pumps: Electrokinetic approach

2. Electrokinetic control

- pumping channels use electrokinetic actuation (i.e., EOF) to produce flow
- opposing EOF streams are combined into one pressure-driven stream
- pressure-driven flow is directed to the separation channel
- pumping pressures ~10 bar (in separation channel)
- liquid flow rates 50-80 nL/min

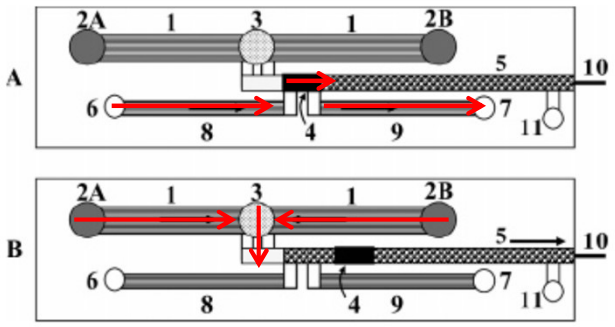
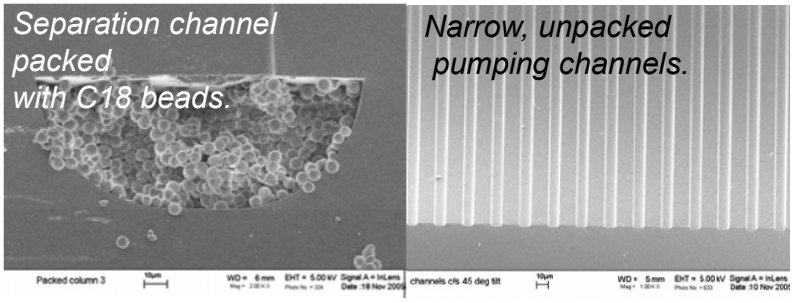


Figure 1. Schematic representation of a microfluidic LC system. Key: (1) pumping channels; (2A) and (2B) eluent inlet reservoirs; (3) eluent outlet reservoir; (4) double-T injector that contains the sample plug; (5) separation channel; (6) sample reservoir; (7) sample waste reservoir; (8) sample inlet channels; (9) sample outlet channels; (10) ESI capillary emitter; (11) LC waste reservoir. (A) Sample loading; (B) sample analysis. Note: arrows indicate the main flow pattern through the system.

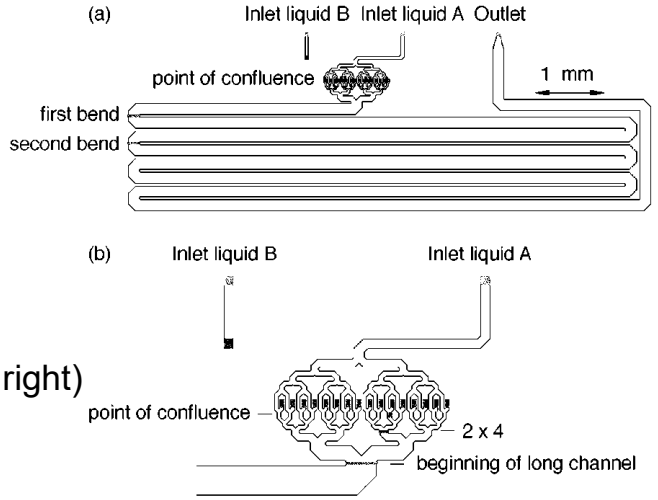


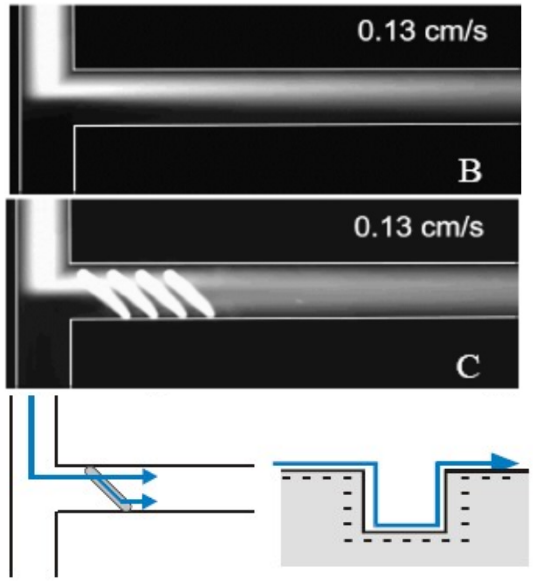
Lazar, I., et al., Anal. Chem. 2006, 78, 5513-5524.

On-chip mixing

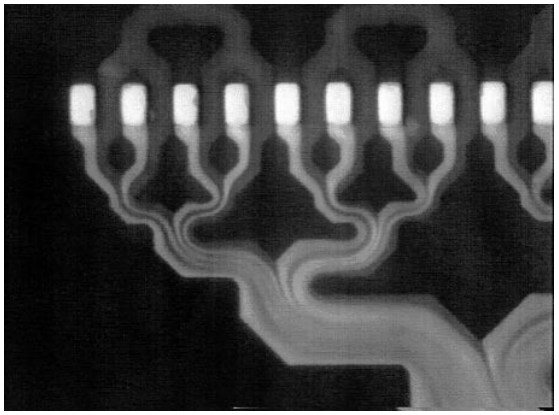
- active mixing → creation of local turbulent flow
  - e.g., by magnetic stirring
- passive mixing (more common)

  → shortened diffusion distance, altered stream lines
  - e.g., with grooves (picture below) or split-and-combine structures (picture on right)





Johnson, T.J., et al., Microfluidic Passive Mixing Structures, at the Electrochemical Society Meeting.

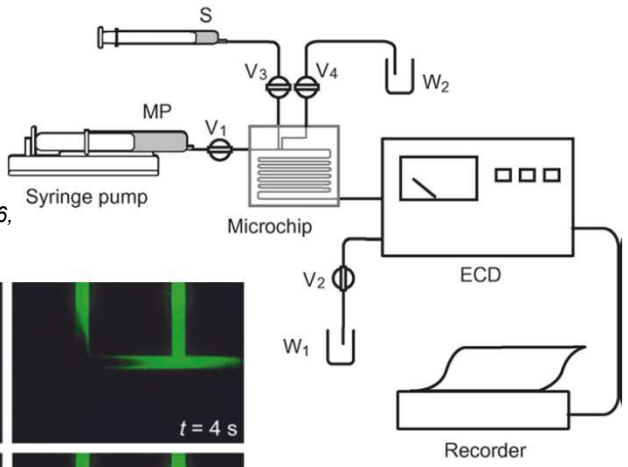


Bessoth, F., et al., Anal. Commun., 1999, 36, 213–215.

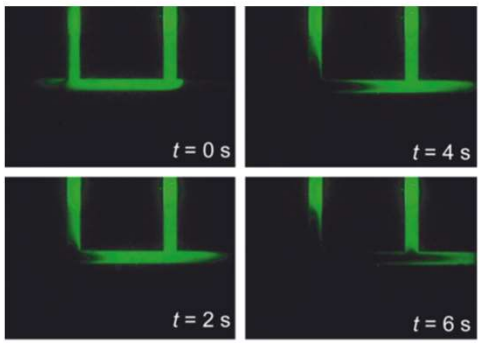
Valving with pressure-driven flow

- Much more demanding than valving of electrokinetic flow, on-chip valving typically achieved by adjusting the fluidic resistances of the intersecting channels

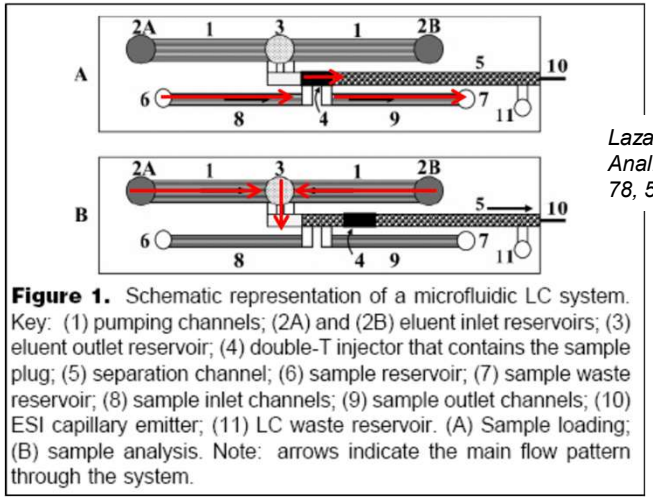
Off-chip external pumps and valves + on-chip injection



Ishida, A., et al., J. Chromatogr. A 2006, 1132, 90–98.



On-chip integrated pumps + on-chip injection



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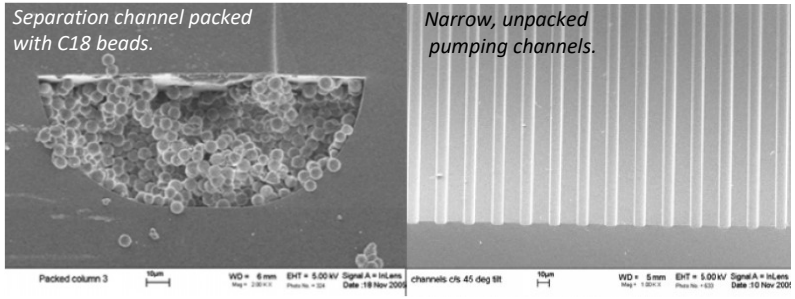
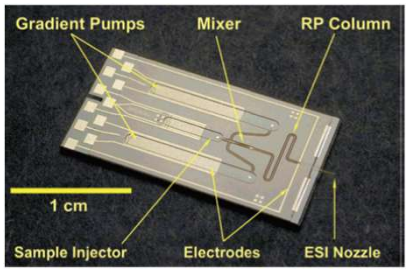
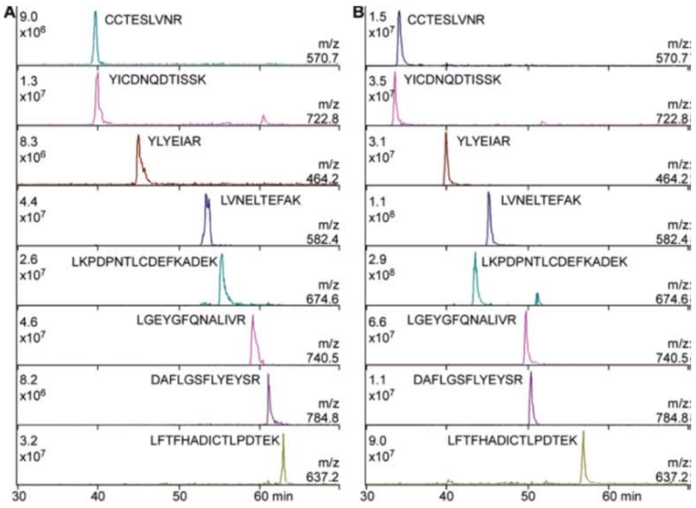


Examples of chromatographic separations on chip

Xie, J., et al., Anal. Chem. 2005, 77, 6947-6953.

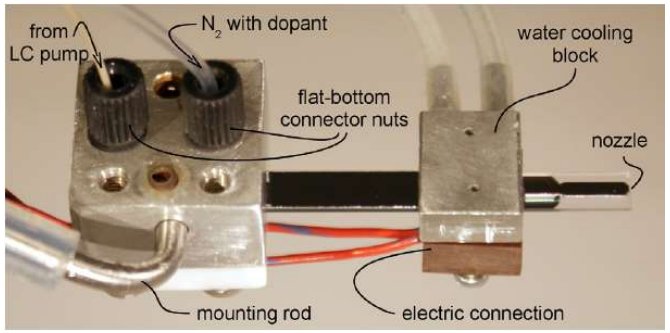
- on-chip pumping and mixing
- on-chip injection
- separation channel packed with C18 beads
- analysis of complex peptide samples



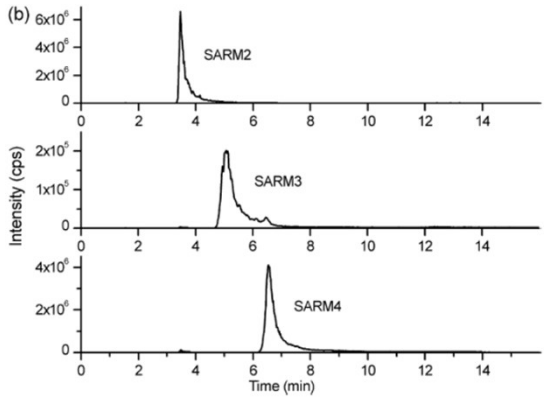


Haapala, M., et al., Anal. Chim. Acta 2010, 662, 163–169.

- off-chip pumping and mixing
- off-chip injection
- separation channel packed with C18 beads
- analysis of novel doping agents (SARMs)



Note the interdepency of separation efficiency vs. analysis time



Comparison of the common fluid propulsion mechanisms in microfluidics

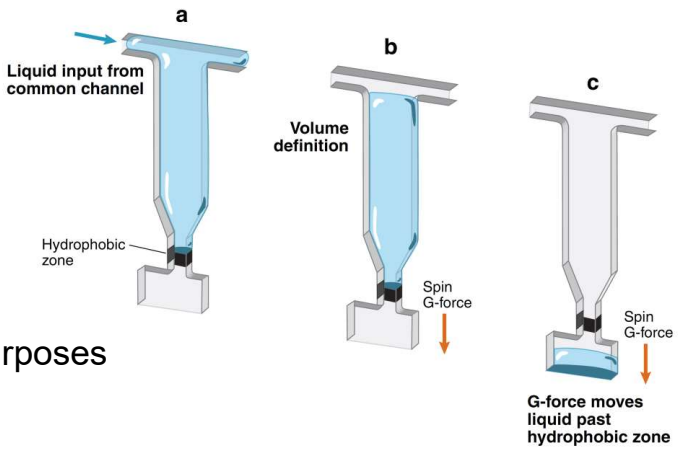
LC, GC CE

Fluid propulsion mechanism				
Comparison	**Centrifuge**	**Pressure**	**Acoustic**	**Electrokinetic**
Valving solved?	Yes for liquids, no for vapor	Yes for liquids and vapor	No solution shown yet for liquid or vapor	Yes for liquids, no for vapor
Maturity	Products available	Products available	Research	Products available
Propulsion force influenced by	Density and viscosity	Generic	Generic	pH, ionic strength
Power source	Rotary motor	Pump, mechanical roller	5 to 40 V	10 kV
Materials	Plastics	Plastics	Piezoelectrics	Glass, plastics
Scaling **	L^3	L^3	L^2	L^2
Flow rate	From less than 1 nl s^{-1} to greater than 100 µl s^{-1}	Very wide range (less than nl s^{-1} to liter s^{-1})	20 µl s^{-1}	0.001–1 µl sec^{-1}
General remarks	Inexpensive CD drive, mixing is easy, most samples possible (including cells). Better for diagnostics	Standard technique. Difficult to miniaturize and multiplex	Least mature of the four techniques. Might be too expensive. Better for smallest samples	Mixing difficult. High voltage source is dangerous and many parameters influence propulsion, better for smallest samples (HTS)

**L=characteristic length corresponding to capillary diameter

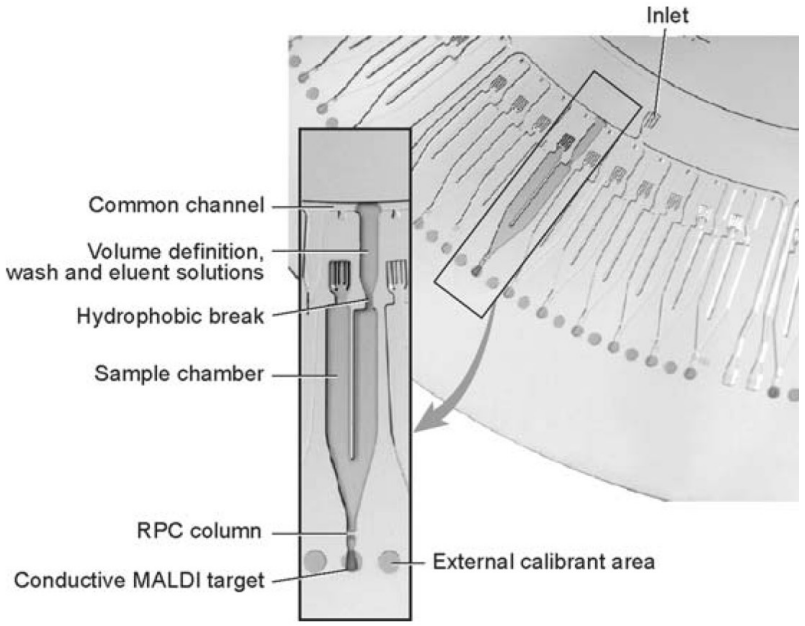
Madou, M., et al., Annu. Rev. Biomed. Eng. 2006. 8:601–28 (review).

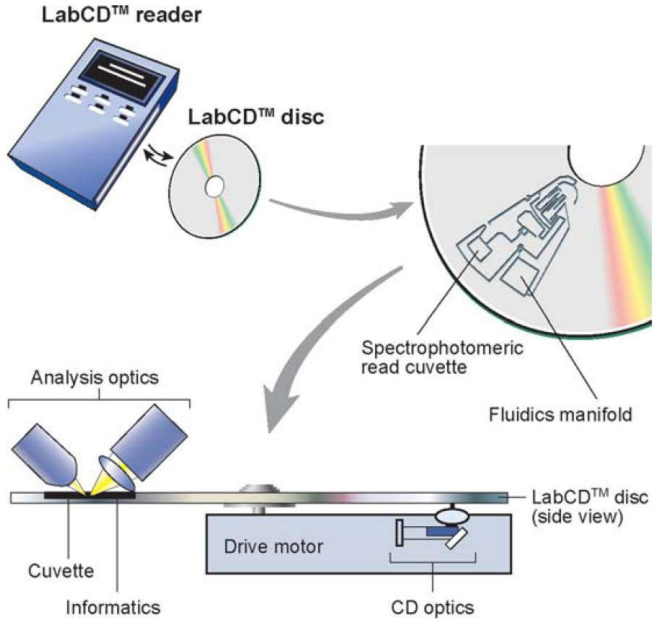
Extra material: Centrifugal Lab-on-a-CD



- A whole different field of research
  - exploits centrifugal forces for fluid propulsion
  - hydrophobic/hydrophilic barriers for valving purposes

- Can also be used for LC applications



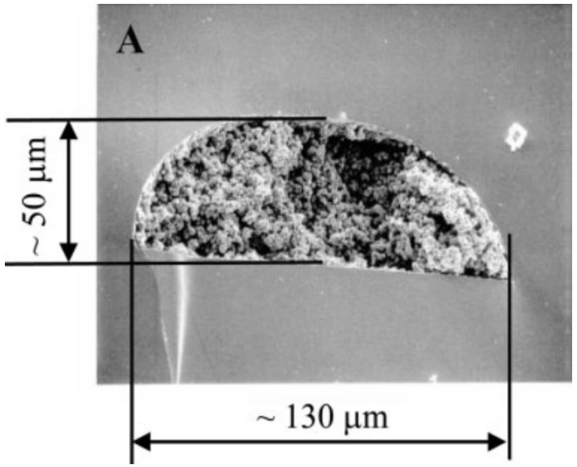


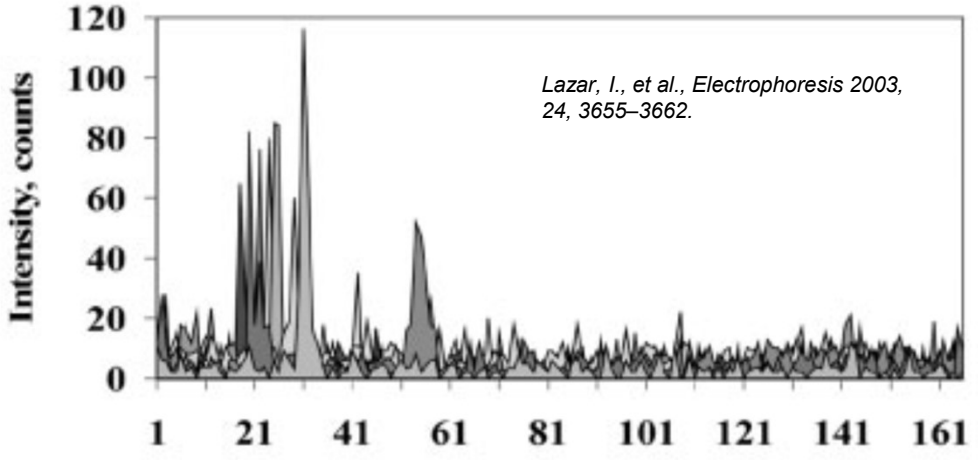
Madou, M., et al., Annu. Rev. Biomed. Eng. 2006, 8, 601-628.

Extra material:
Capillary electrochromatography on chip – A mixture of CE & LC

Packed Channel CEC

- glass channel packed with polymer monolith
- Actuation by electrokinetic flow → Thus, primarily considered as an electrophoretic separation technique
- Separation is due to both electrophoretic mobility differences of sample components (same as in CE) and chromatographic interaction between the sample components and the solid phase (same as in LC)





To wrap-up:
Microchip CE has many advantages over microchip LC (from design and microfabrication perspective)

- CE separation efficiency mostly relates to electric field strength
  - does not suffer from scale-down
  - no need for postprocessing (packing of the stationary phase)
  - but the surface must be inherently charged

- Electroosmosis as the fluid propulsion mechanism has low back-pressure
  - no need for high pressures or pressure-tight interfaces
  - no need for complex macro-to-micro interfacing (with external pumps)
  - variety of materials and techniques available for chip fabrication
  - but is often less stable/repeatable compared with pressure driven flow

Background literature

- electrophoretic separation on chip
  - *Wu, D. and Qin, J., J. Chrom. A 2008, 1184, 542-559*
  - *Karlinsey, J.M., Anal. Chim. Acta 2012, 725, 1–13.*
- chromatographic separation on chip
  - *deMello, A., Lab Chip, 2002, 2, 48N–54N*
- overview on optical and electrochemical detection
  - *Bashir, R., et al., Adv. Drug Deliv. Rev. 56 (2004) 1565– 1586*
- microchip technology in mass spectrometry
  - *Sikanen, T., et al., Mass Spectrom. Rev. 29 (2010) 351-391*

Extra: To Distinguish the Chromatographic Techniques (LC, GC) From Capillary Electrophoretic (CE) Techniques

- two different SEPARATION PRINCIPLES
  - CE: separation of the sample components is due to their electrophoretic mobility differences (because of their different size and charge)
  - GC/LC: separation of the sample components is due to differences in the amount of interactions with the stationary phase (because of their different hydrophobicity)

- FLUID PROPULSION mechanisms also differ between these techniques
  - CE: fluid propulsion is due to electrokinetic flow
    - potential difference over microchannel having (negatively) charged surface induces EOF because of cation (+) motion toward the opposite (-) electrode (Coulomb force)
  - GC/LC: fluid propulsion is due to pressure-driven flow
    - off-chip external pumps or on-chip integrated pumps; sometimes centrifugal pumping

- both techniques include several different SEPARATION MODES
  - CE: zone electrophoresis, isoelectric focusing, micellar electrokinetic chromatography…
    - capillary ZONE electrophoresis is the by far most common method for microfluidics
  - GC/LC: discriminated by the state (gas or liquid) of the mobile phase
    - the state of stationary phase: either liquid or solid in GC; always solid in LC