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Integrated Microfluidics Workshop
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Outline (may not get to it all)

- Introductions
- Overview of microfluidics
  - Why microfluidics?
  - Miniaturization and portability?
- Fluid flows in chips
  - Simple fluid mechanics
  - Multiphase flows in chips
    - Two-phase extraction
    - Droplets
  - Driving, metering, and controlling fluid flows
  - Flow cytometry and flow sorting
- Photopatterned materials in microfluidic chips
- Chip-world interfacing
  - LIF detection
  - Fittings, ports, and manifolds

Who am I? Why am I here?

Robert Meagher, Ph.D. (Barron, 2005)

- Technical Staff at Sandia National Laboratories, Livermore, CA, USA
  - Microfluidics and miniaturization for DNA, proteins, and bacteria
  - Previous work with electrophoresis (CE and chip) and two-phase flow in chips
  - Background in chemical process engineering
- Disclaimers
  - I am not here to endorse any companies, brands, or commercial products on behalf of myself, CASSS, Sandia National Laboratories, the United States Government, or any other entity.
  - I will mostly refrain from mentioning brand names during the presentation. If you would like more information on commercial sources for products I mention, please ask.
  - Many of my examples will be drawn from work done by myself or others at Sandia; this in no way is meant to discount the fine work done by many research groups around the world!

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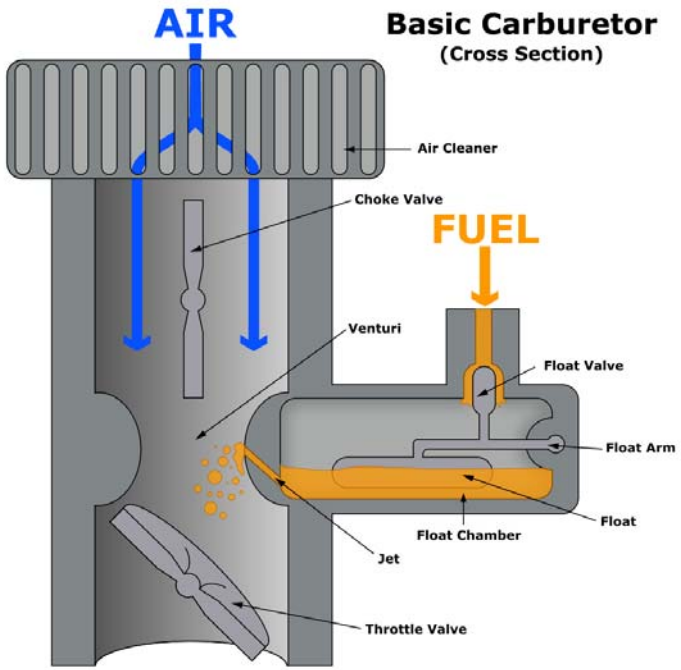
What is Microfluidics

- Depends who you ask!
- “Lab on a Chip”
  - Usually implies microchannels etched, embossed, molded, etc in a monolithic substrate (glass, silicon, plastic, etc)
- More generally, microfluidics comprise any system for controlled flow/access/interrogation of fluids (liquid or gas) in channels or tubes where at least one dimension is <1 mm.
  - But not always…
  - May include “lab on a chip” devices, capillaries, needles, nozzles, fittings, etc.

Are these microfluidics?

Airbrush (www.wikipedia.org)



Carburetor (www.wikipedia.org)

Yes they are, but mostly we will focus on microfluidics to enable biological assays or bio research.
Key point: there is a good amount of basic scientific knowledge as well as technical art in microfluidics that pre-dates the modern “lab-on-a-chip” era (~early 1990s - present)

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Thoughts on miniaturization

- Microfluidics is one route toward miniaturization of analyses
  - Microfluidic channels are inherently scaled for analyzing small volumes (nL – few µL)
  - Reagent savings: cost of expensive reagents (*e.g.* antibodies or enzymes, isotopically labeled chemicals) become negligible on a per-assay basis
  - Small volumes are appropriate to analyzing small numbers of analytes (*e.g.* a few molecules or cells).
  - Miniaturization of channels can lead to reduced time for analysis, *e.g.* electrophoresis in ~ 1 minute in a 2-cm microchannel vs ~20 minutes in a 40-cm capillary; also potentially reduced time for processes based on diffusion, mixing, etc.

- Miniaturization of analysis by microfluidics is also a route toward miniaturization and automation of *instrumentation*.
  - *E.g.* point-of-care diagnostics, portable analytical devices, *etc.*
  - Reduced footprint for laboratory equipment

- Smaller ≡ Faster, Cheaper, Better?
  - Not necessarily! Sometimes slower, less sensitive, less precise, clogs more easily, wastes more reagent, *etc* than a well-engineered piece of “macro” scale instrumentation.

Not every biological analysis needs to be ported to a chip format!

- Biology research (especially with cells) tends to be very dependent on an individual lab or researcher's technique.
  - Thus biology researchers tend to be inherently skeptical of "new" and "better" ways of doing "old" things.
- If there is an easy, commercially available kit, device, or instrument for performing a certain assay or analysis, don't bother to "microfluidize" it
  - Especially if a modest increase in speed or decrease in cost is offset by a decrease in sensitivity, reliability, etc.
- Biologists whole-heartedly embrace new techniques/technology when it provides something that was previously not feasible, not just moderate improvements
  - E.g. chip-based Sanger sequencing has not displaced capillary-based sequencing, but next-gen sequencing techniques (which are also "microfluidic") have enabled new types of research.
- Many simple, every-day tasks in the biology lab actually involve numerous steps that require attention to detail, and intermediate "QC" analysis and decision points requiring human evaluation
  - Difficult to engineer automated solutions for these.

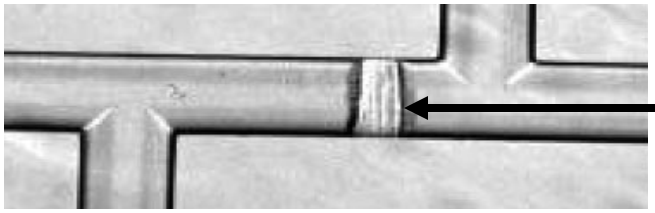
When does microfluidics make sense?

- In the academic / research lab:
  - When no current technique or kit seems to do quite what you want
  - When you want to take advantage of phenomena only available or practical in small scale devices, *e.g.* certain electrokinetic phenomena or fluid flow patterns.
  - When there is a clear benefit to integrating multiple steps of an analysis into a single device.
- Commercial possibilities: microfluidics may enable certain low-cost, portable- or point-of-care diagnostics or analyses, which can be mass-produced and widely distributed.
  - Often talked about, and some great technology demonstrations, but as yet no big commercial blockbusters.
  - For portable devices: how easy is easy enough? How unskilled is the unskilled user? How resource-poor is resource-poor?
  - Technology alone is not the only driver for success in commercialization: what is the path for clincal acceptance or approval; will the outcome of a point-of-care analysis affect a clinician's course of treatment or prognosis for a patient, *etc.*

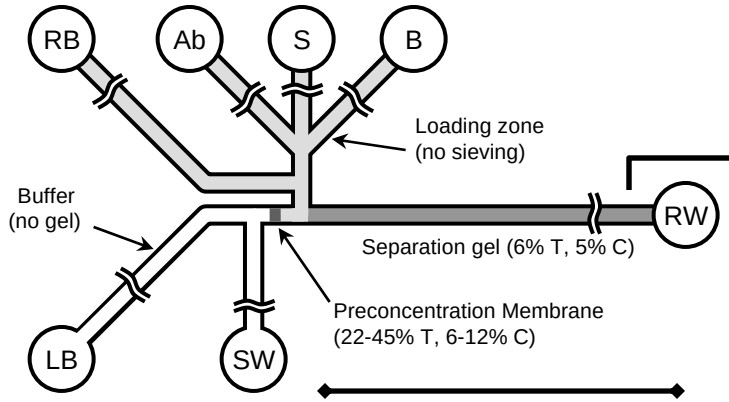
Microfluidics for compact instrumentation?

- Example: microfluidic device with photopatterned hydrogel membrane for sample preconcentration and reaction/labeling, followed by electrophoretic separation

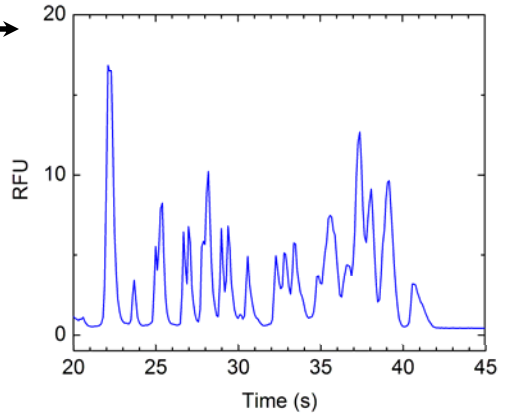
Image from Herr *et al PNAS* 2007



Hydrogel membrane
Characteristic dimension ~ 100 µm
Analytes (proteins & antibodies, DNA, etc) can be concentrated >1000-fold, mixed, incubated, etc. and then separated by zone electrophoresis
A good example of a “micro” (or “nano”) element enabling a more sensitive analysis



Separation channel
Characterisic dimension ~ 2 cm



Separation of DNA ladder after preconcentration at membrane

Meagher 2009

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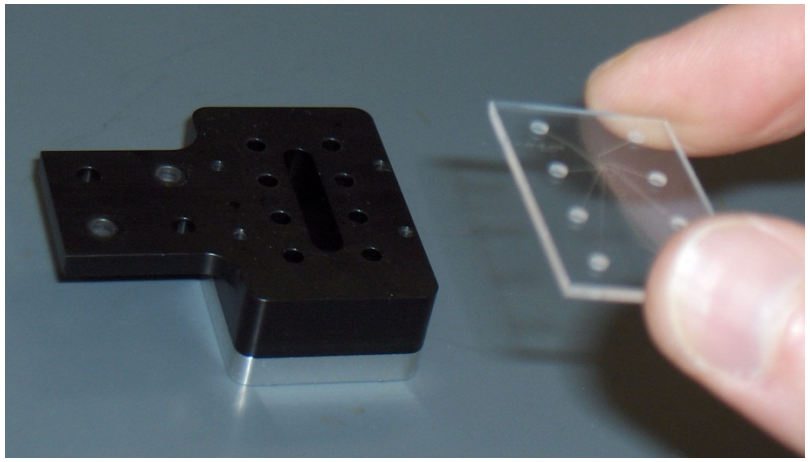
Actual experimental setup for DNA preconcentration/separation experiment

Chip holder (manifold)
~ 5 cm x 5 cm x 2 cm

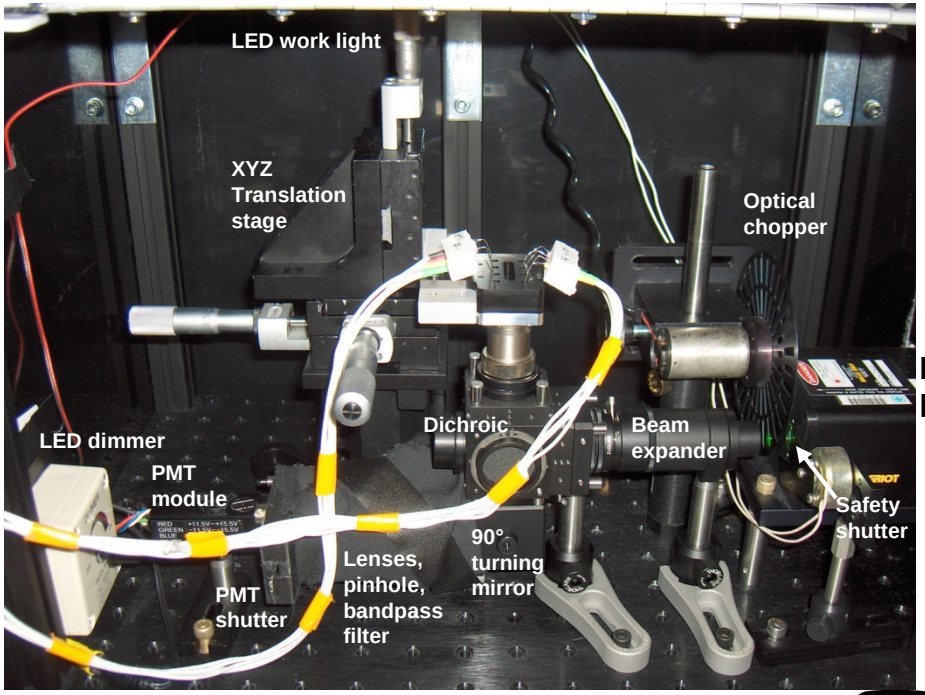


Glass chip
~ 2 cm x 4 cm

Electrode "positioning blocks"

Chip in manifold with Pt electrodes and 40X focusing objective, CD ~ 5 cm

electrode leads (insulated for HV) 60 cm



Confocal LIF detection setup – "compact" setup with conventional 25 mm optics ~ 45 cm x 30 cm x 30 cm

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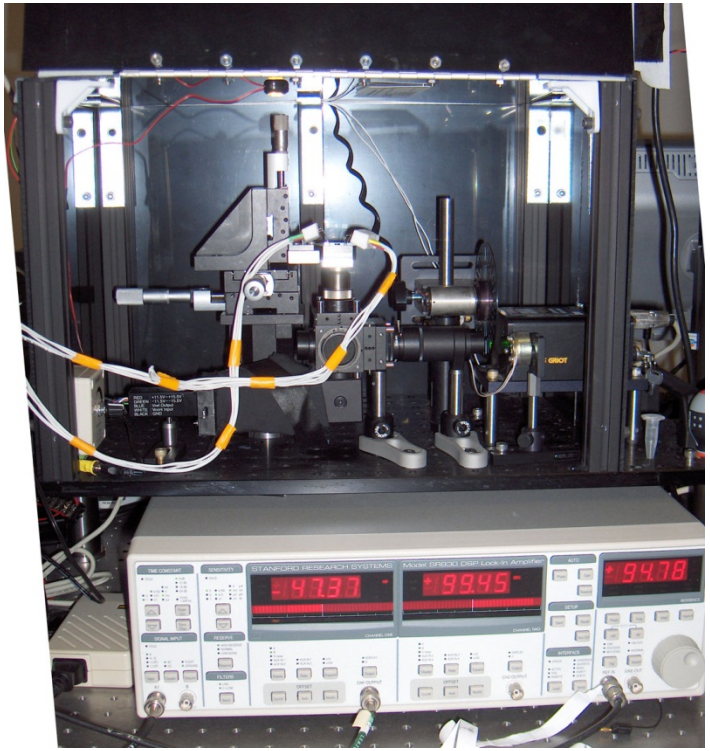
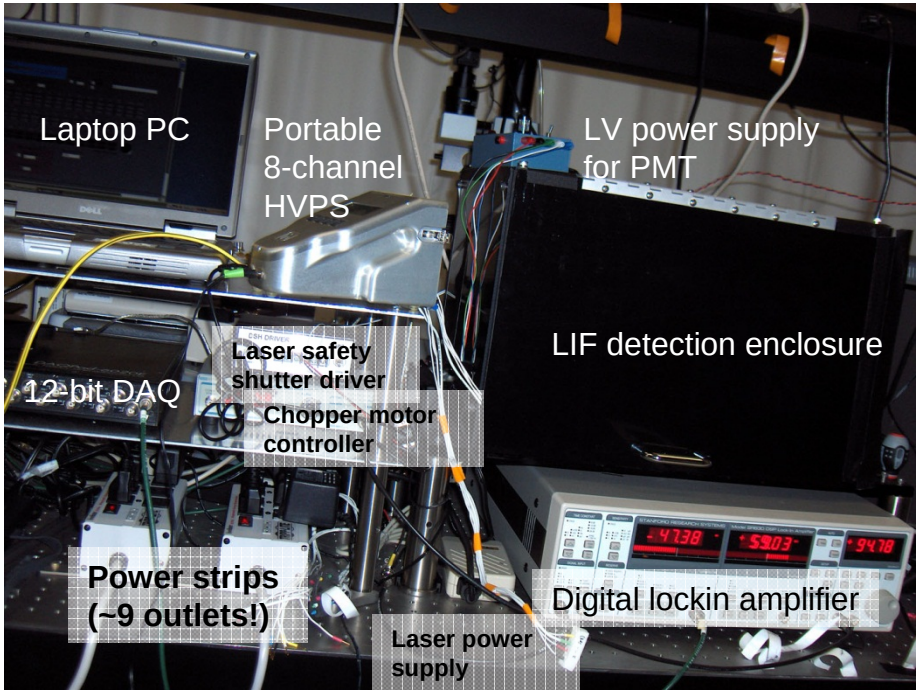


Detector on optical breadboard in light-proof enclosure and digital lock-in amplifier
~ 60 cm x 45 cm x 45 cm



Supporting instrumentation for membrane preconcentration / chip electrophoresis experiment
Mounted on optical table
~ 120 cm x 60 cm x 60 cm

Not pictured: wet lab bench for filling chip, etc.

For routine, day-to-day lab work and chip assay development – this is *less* miniaturized than a typical commercial CE instrument!

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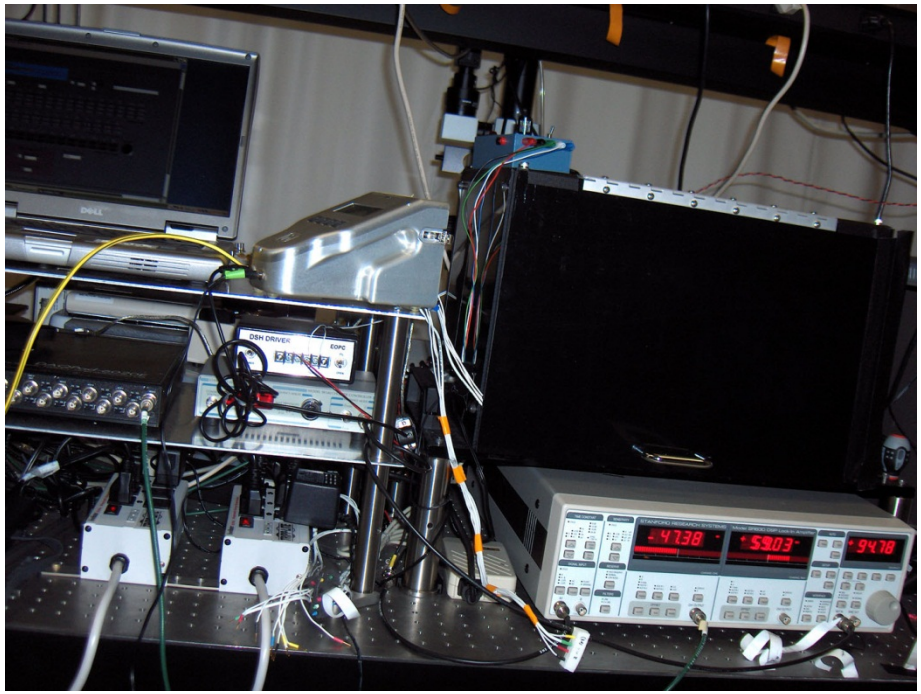
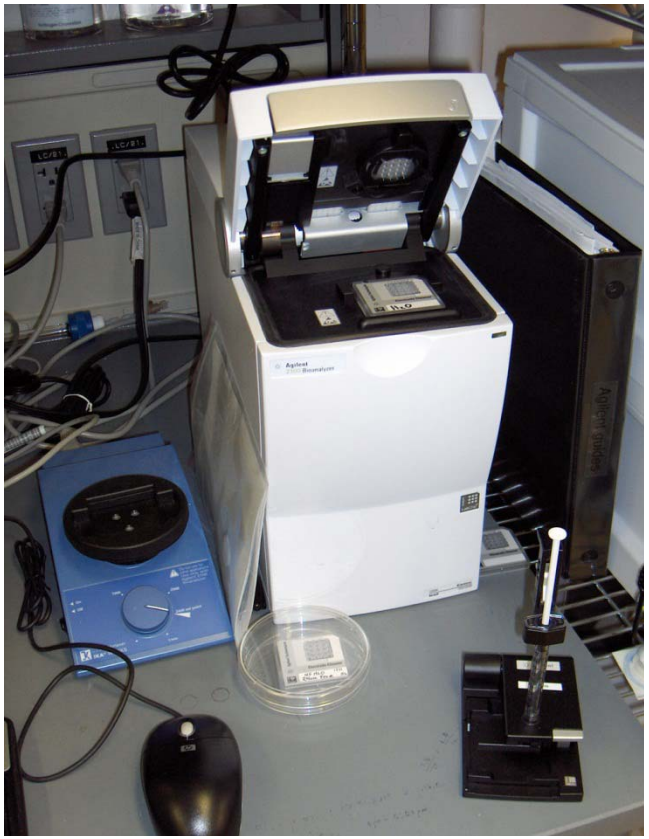
- With millions of dollars of additional engineering and packaging, my sprawling setup could *become* a compact, easy-to-use box, available to the mass market at a modest price.



Many people-years $$$$

?



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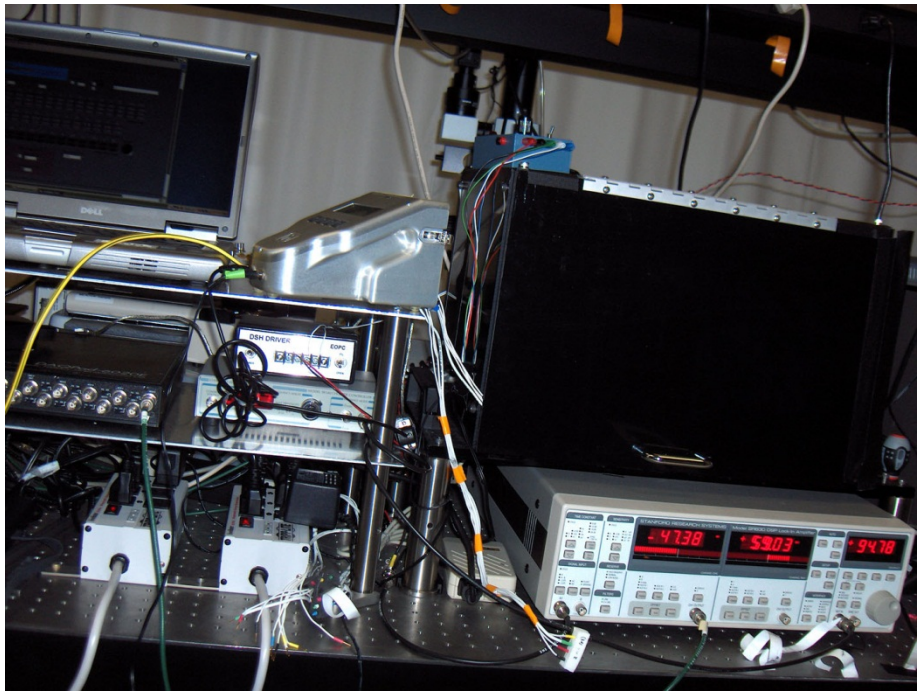
- The commercial instrument doesn’t do what my chip does
- I don’t have millions of dollars and a big engineering team
- But I want results now!
- Hence the sprawling setup is here to stay.



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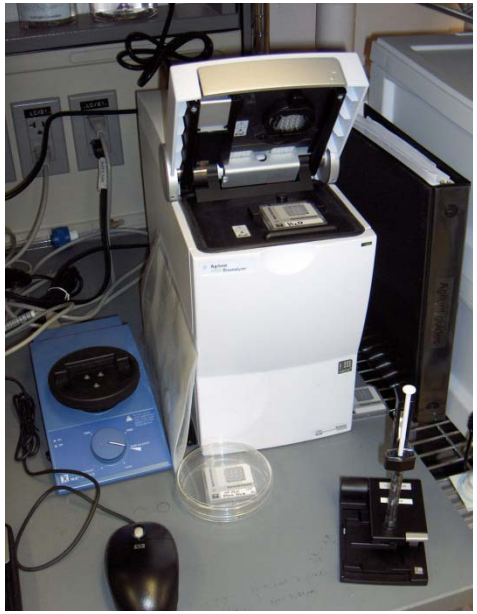
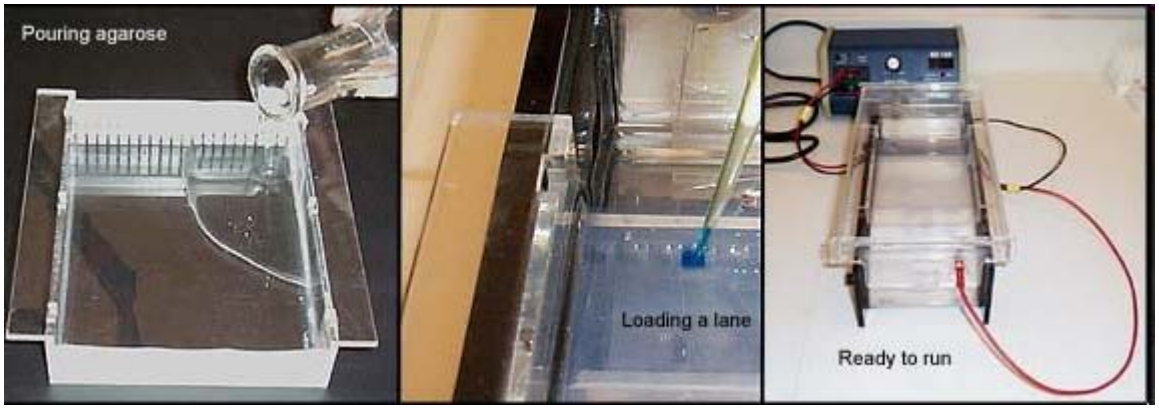
Microfluidics is *not* the only possible solution…

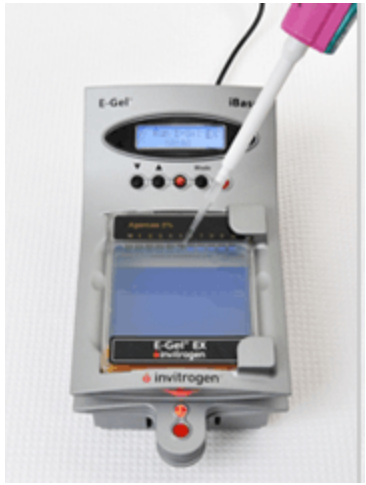


replaces



Picture from Colorado State University
http://arbl.cvmbs.colostate.edu/hbooks/genetics/biotech/gels/agardna.html

But so does…



Different advantages for different applications!

Precast gels are cheap and easy and work every time.

The chip is more sensitive and wastes less sample, unless your objective is to cut out a band…

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And if you did want to purify a band…

Image from MIT, http://web.mit.edu/7.02/virtual_lab/RDM/RDM1virtuallab.html

Lab-on-a-Chip solution

Caliper LabChip XT

Non-chip solutions (easier than a razor blade, different from chip in ease, expense, capacity, precision, *etc)*

Invitrogen E-gel SizeSelect™ Gels

Also: SPRI beads (non-gel, non-electrophoretic size-selective capture of DNA)

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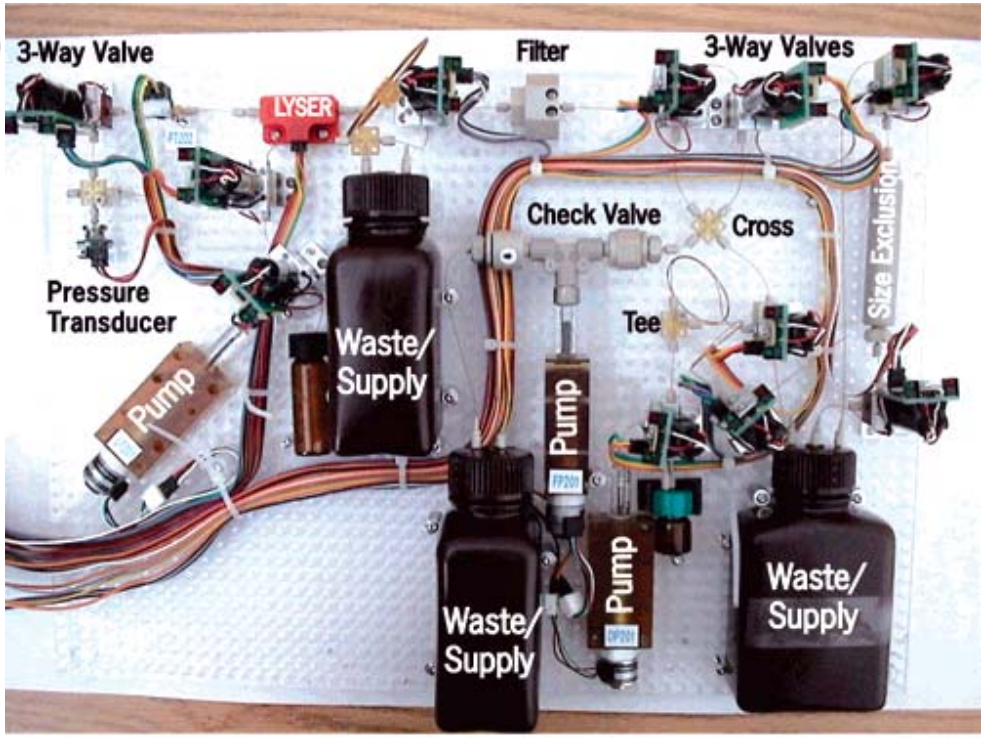
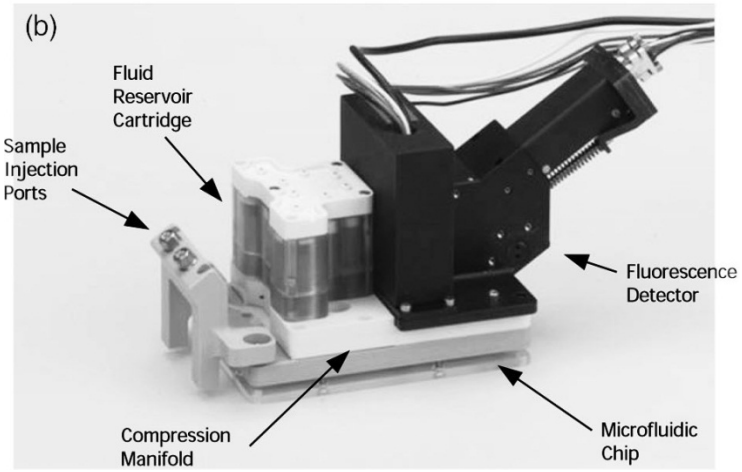
“Integrated Microfluidics” is not necessarily “Lab on a Chip”

(a)





Example: miniaturized (but not monolithic) sample-prep train for automated bacterial spore concentration, lysis, and fluorescent labeling of proteins *prior to* chip electrophoresis for protein separations

(a) from Stachowiak *et al*, *Anal. Chem.* 2007, (b) from Fruetel *et al*, *Electrophoresis* 2005

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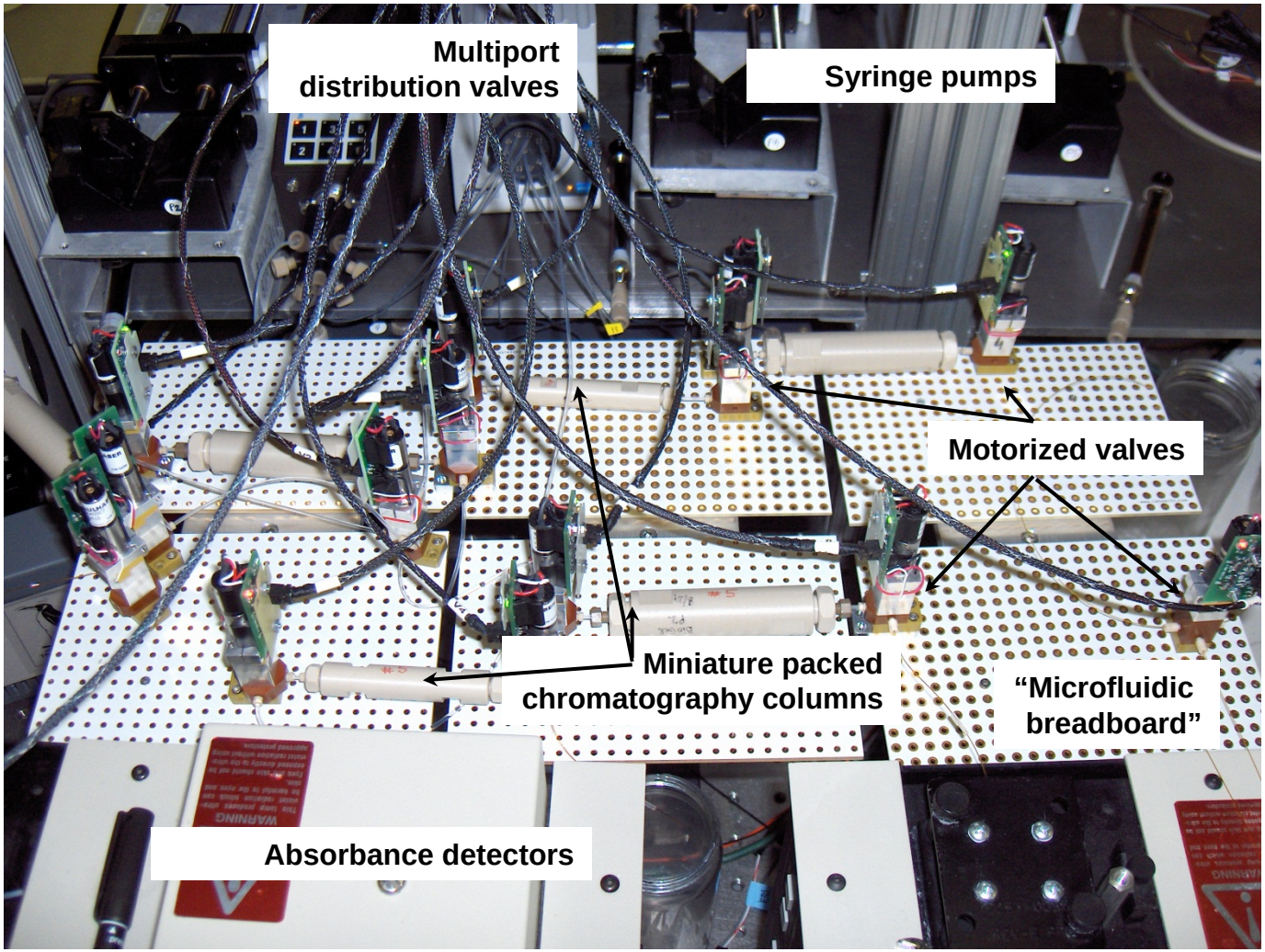
Microfluidics without chips



Courtesty G. Chirica, SNL

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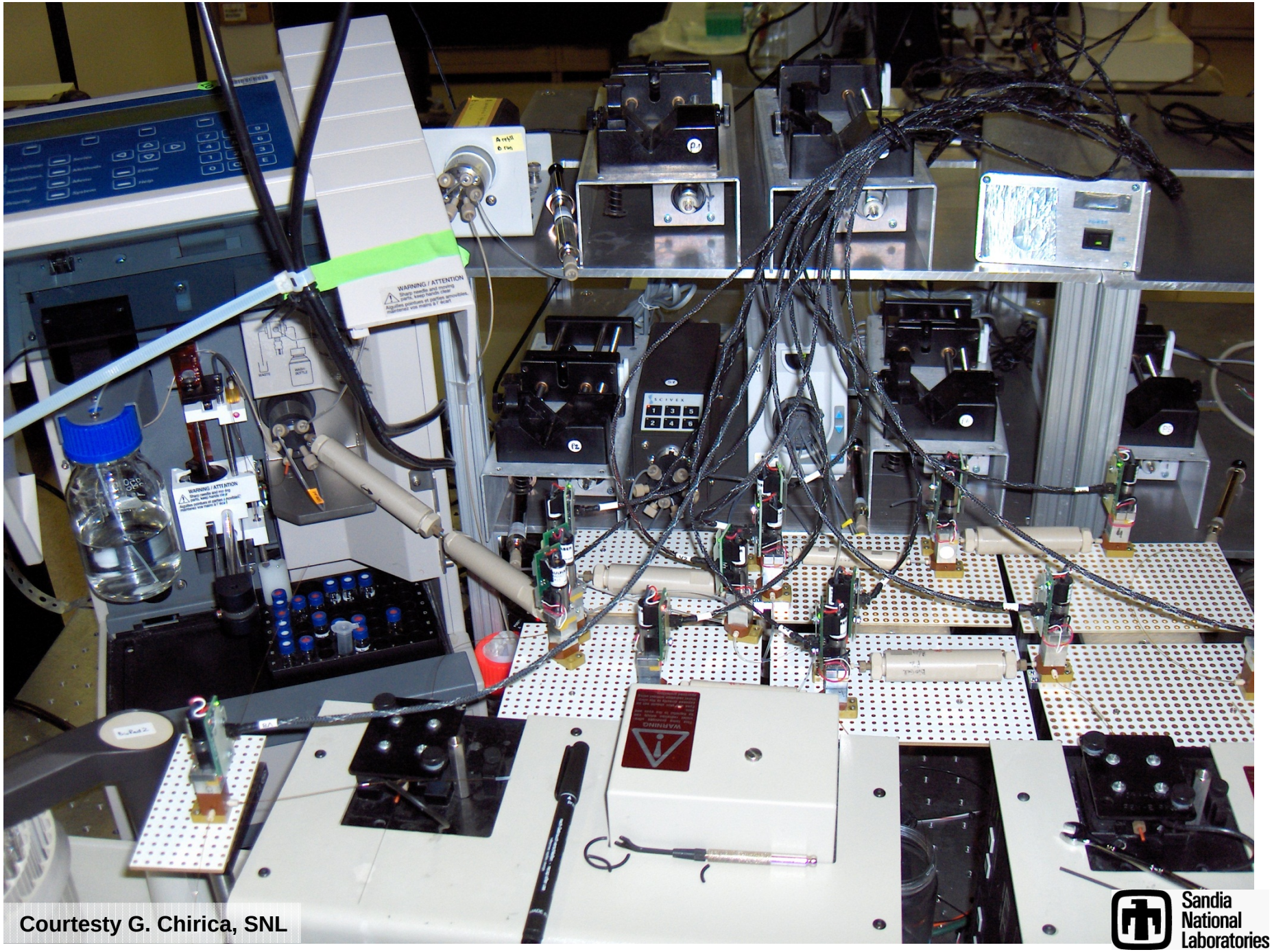

Courtesy G. Chirica, SNL

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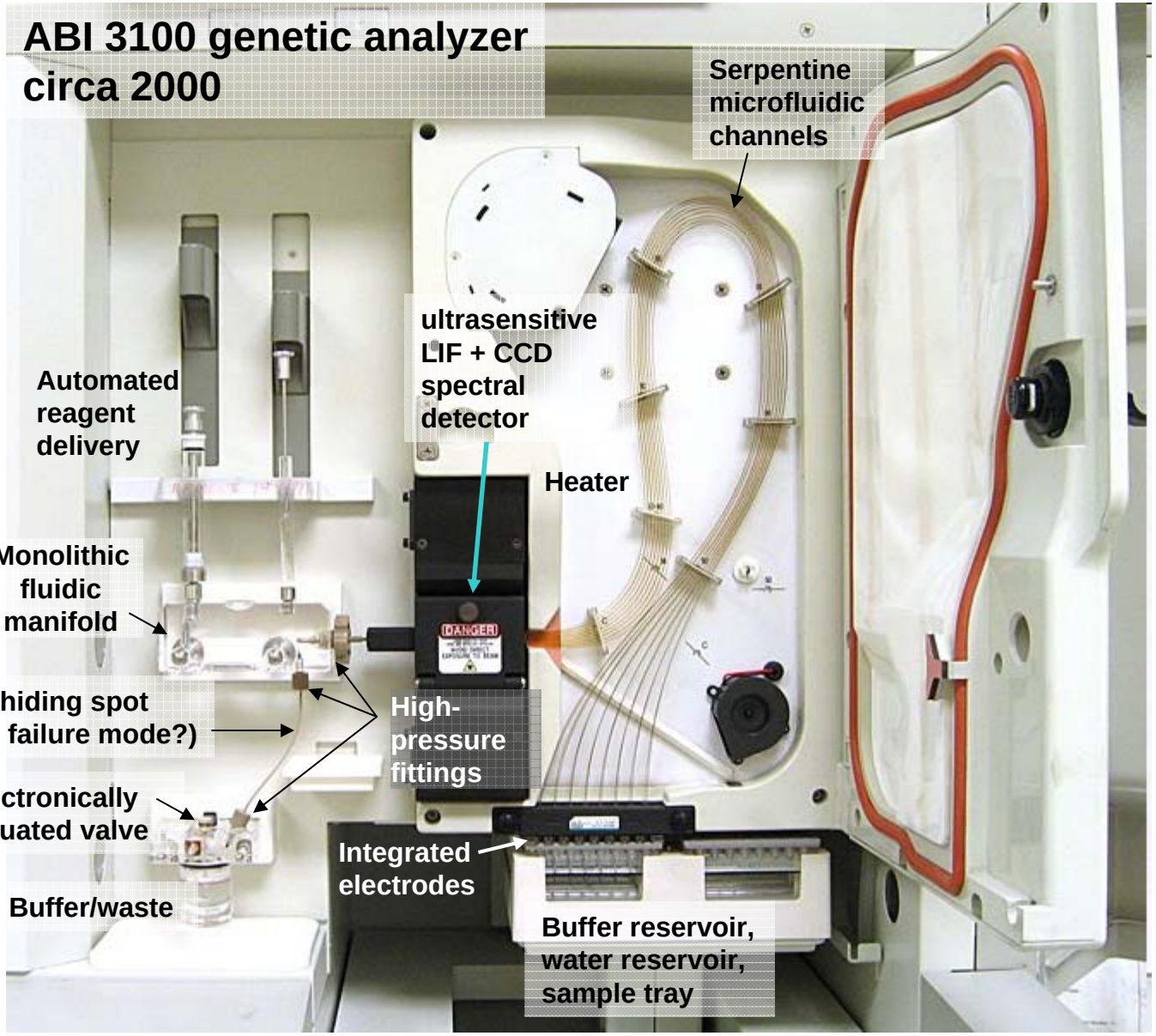
Integrated microfluidics?

~ 100 cm x 60 cm x 150 cm



Inside the box:
HV power supply
Ar-ion gas laser
Cooling fan
Heater element
Syringe pump driver
XYZ motion controller
for buffer/water/
sample trays
DAQ hardware
Computer interface
hardware

www.wikipedia.org

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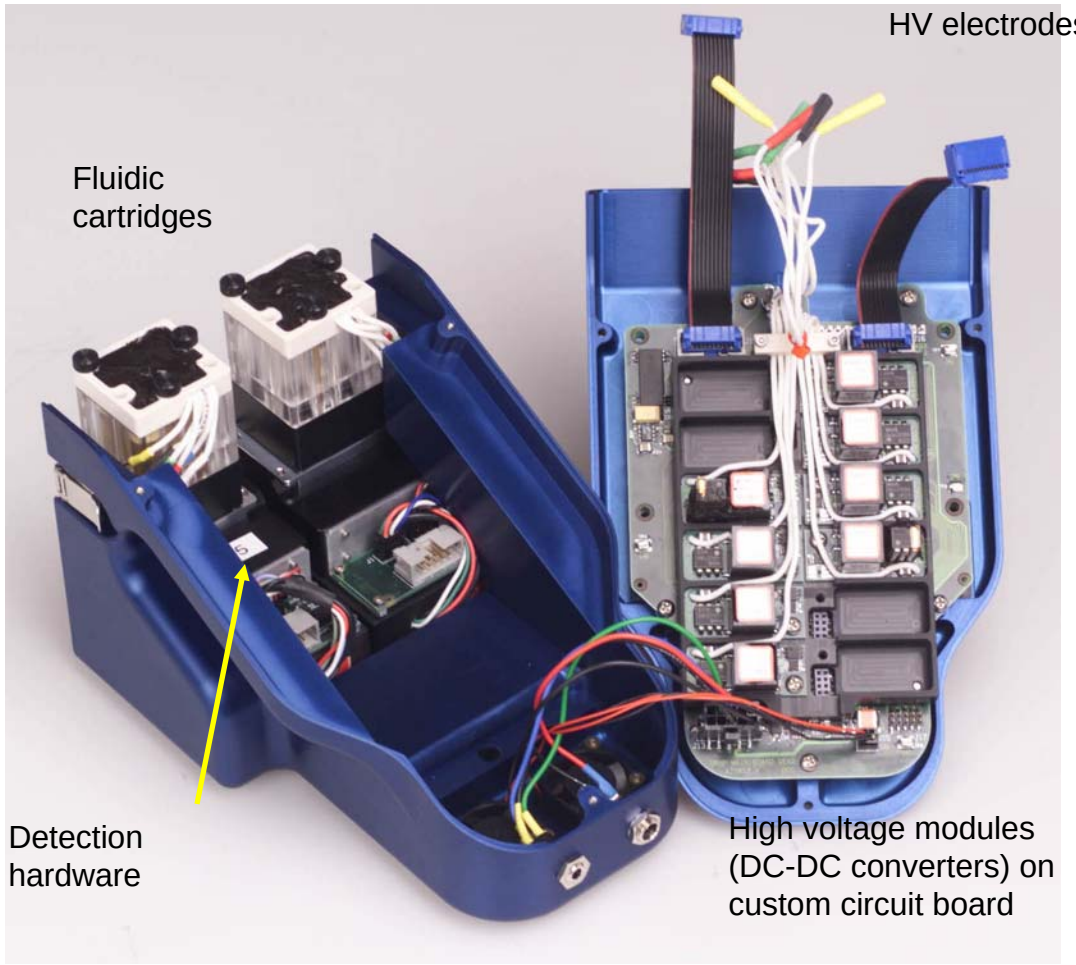
Making microfluidics portable (or at least smaller)



Hardware designed and built by: Ron Renzi, Jim Van De Vreugde, Mark Claudnic, Jerry Inman *et al*

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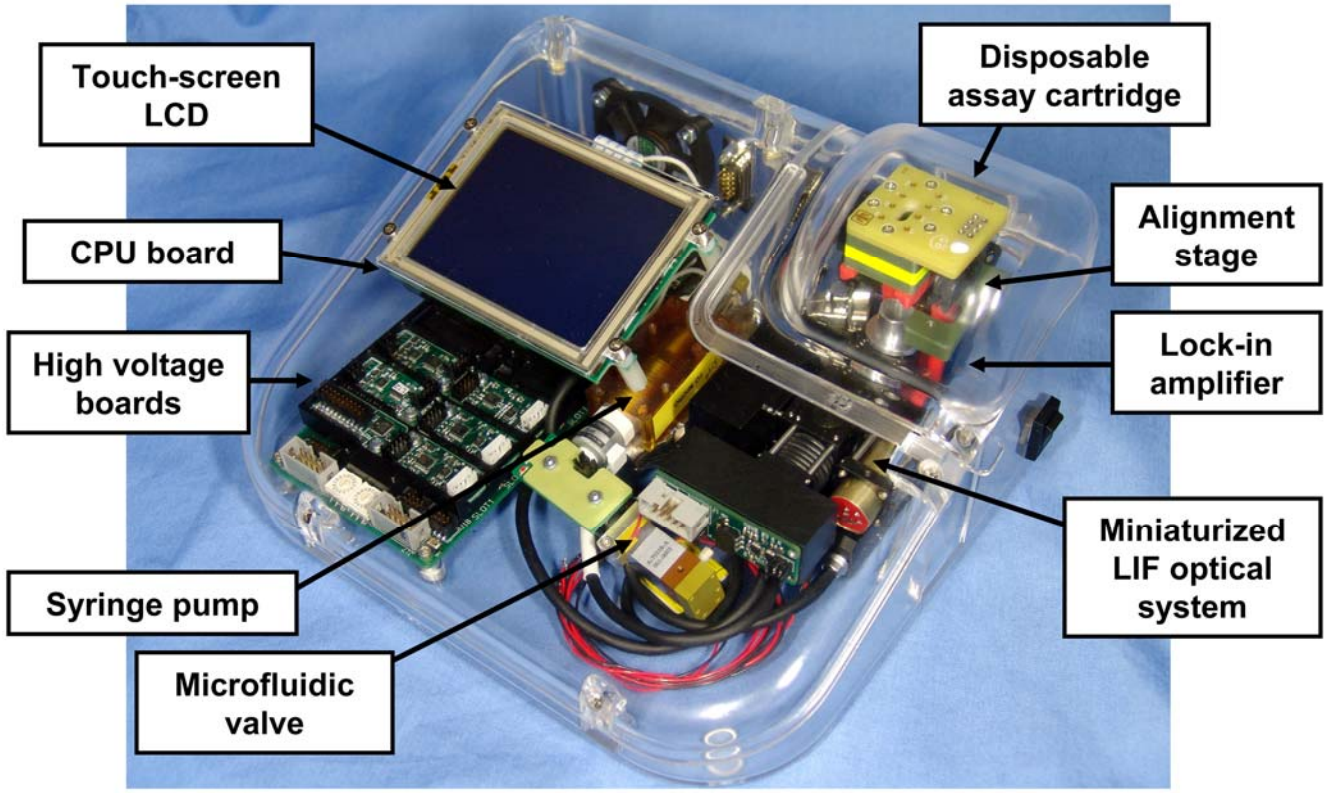
Portable instrument for microfluidic immunoassay



Hardware designed and built by: Ron Renzi, Jim Van De Vreugde, Mark Claudnic, Jerry Inman *et al*

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Pressure-driven flows in microfluidics

(as opposed to electrophoresis/electrokinetics)

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Reynolds number: relative importance of inertial and viscous forces

$Re = du\rho/\mu$

d = characteristic length scale (typically depth or width of channel)
u = velocity scale (typically bulk or average velocity)
ρ = density
μ = viscosity

Re < 2100 – flow is fully laminar ("easy" to model – Navier-Stokes equation for incompressible Newtonian fluids)
Re > 4000 – flow is fully turbulent ("hard" to model in detail, but general characteristics are well known.)

Which *d* to use?

For circular channels – use the diameter.
For channels of low aspect ratio (width >> depth) – use the depth
For channels of moderate aspect ratio (*width ~ depth*) – calculate an effective hydraulic diameter

d_H = 4 × cross-sectional area / wetted perimeter

Within laminar regime:

***Re* << 1:** "Stokes" or "creeping" flow: $\mu \nabla^2 \mathbf{v} = \nabla P$

velocity and pressure fields respond quickly to changes in pressure
Inertial effects are insignificant - flow stops "instantaneously" when pressure is removed

***Re* >> 1 (but not turbulent!):** "Inviscid" flow: $\rho\, \mathbf{v}\cdot\nabla\mathbf{v} = -\nabla P$

Inertial effects are dominant, no internal viscous dissipation of energy – this underlies the familiar *Bernoulli's equation* (interconversion of kinetic energy, potential energy, and pressure)

Harder to model except in special cases, *e.g.* irrotational flow (not in enclosed channels);

Near solid surfaces there is transition from "inner" or "boundary layer" flow (where viscous forces matter) and "outer" or "free stream" behavior which are purely inertial.

Sample calculation of Reynolds number for water in a microfluidic channel:

d = 50 e-06 m (50 µm)

q = 1 µL/min; average velocity u = 8.5 e-03 m/s (8.5 mm/s)

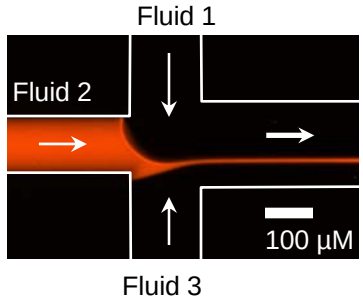
ρ = 1000 kg/m3

m = 0.001 Pa*s (1 cP)

Re = (50 e-6)(8.5 e-3)(1 e3)/(1 e-3) = 0.42

This is definitely laminar, closer to the "creeping" flow than "inviscid" flow regime, but there will be some inertial effects, *e.g.* it will take some time for the fluid to slow down and stop once pressure is released.

Image: Meagher *et al*, *MTAS* 2008



Q: In this 50-µm channel with flowing water, what velocity is required to reach Re = 100 ("inviscid" flow)? Re = 10,000 (turbulent)?

A: Re = 100 requires u = 2 m/s (pretty fast!), or q = 236 µL/min.
Re = 10,000 requires u = 200 m/s (very fast!), or q = 23.6 mL/min

The Reynolds number allows us to draw analogies between familiar, macroscopic flows where we have everyday intuition, and microscale flows which may be less familiar.

For our Re = 0.42 example, an equivalent flow would be drinking a milkshake (700 cP)* through a straw 5 mm in diameter, at 1 mL/s (~5 cm/s).

*our intuition begins to break down because many familiar high-viscosity fluids (like these milkshakes) are non-Newtonian, *i.e.* the viscosity is a function of shear rate and sometimes history.

Milkshake photo: microsoft office clip art

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Other important transport parameters

- In situations with diffusive mixing – *Peclet number* describes relative rates of convection (transport by the bulk flow down the length of the channel) *vs* diffusion "across" channel (perpendicular to flow)
  - *Pe* = *uL*/*D* *(velocity* × *length scale / diffusivity)* – choose *L* as length scale relevant for diffusion
  - *D* for macromolecules (*e.g.* proteins) in water is often pretty small, *e.g.* 10^{-10} m^2/s, so *Pe* is often large despite the short length scales (diffusion is slow relative to convection).

- Characteristic time scale for diffusion $t_D \sim L^2/D$
  - Characteristic time for a solute with diffusivity *D* to diffuse across a length *L*
  - Example: for a protein with diffusivity $D = 10^{-10}$ m^2/s, diffusion time for 10 μm is 1 second, diffusion time for 100 μm is 100 seconds.
  - Time to approach "fully-mixed" state by diffusion alone will be several multiples of t_D.
  - *Key point: mixing by diffusion alone over more than a few microns is often a very slow process*

- *Pressure drop* in laminar flow is a strong function of channel diameter or length scale, and also fluid viscosity
  - For circular cross section, volumetric flow rate $q = (\pi/128)\,(\Delta P/L)\,d^4 / \mu$
  - For non-circular cross section of aspect ratio ~1, use hydraulic diameter defined previously.
  - Note especially 4th-power relationship between *d* and ΔP at constant flow rate!
  - In chips – small obstructions or constrictions can lead to large increases in back pressure.

- *Entrance length* for laminar flows, *e.g.* from a wide via hole into a microchannel

$$L_e / d \approx 0.59 + 0.055 Re$$ (from Denn, 1980, cylindrical tube, *Re* < 2100)

  - Characteristic length scale for transition from plug flow to fully developed laminar flow
  - For small Reynolds number flows in chips – this is often negligible
  - For higher Reynolds numbers – this can be 10*d* or more – noticeable for short channels

Pressure drop and flow rate

In a cylindrical channel:

Volumetric flow rate

$$q = \frac{\pi}{128}\left|\frac{\Delta P}{L}\right|\frac{d^4}{\mu}$$

Average velocity

$$u = \frac{1}{32}\left|\frac{\Delta P}{L}\right|\frac{d^2}{\mu}$$

Centerline (maximum) velocity

$$u_c = 2u$$

Sample calculations

For our round channel, with water (μ = 0.001 Pa·s), d = 50 μm, q = 1 μL/min = (0.0167 e-9 m^3/s)
$|\Delta P/L|$ = (0.0167 e-9 m^3/s) (128 / π) (0.001 Pa·s) / (50 e-6 m)^4 = 109 e3 Pa/m
So for a microchannel channel of L = 3 cm, ΔP = 3300 Pa = 0.47 psi

For our other calculated velocties:
Re = 100: q = 236 μL/min: $\Delta P/L$ = 25.7 e6 Pa/m, or 771 kPa (110 psi) for a 3-cm channel (achievable with syringe pump)
Re = 10,000: q = 23.6 mL/min: $\Delta P/L$ = 2.57 e9 Pa/m, or 77100 kPa (11,000 psi) for a 3-cm channel (not easily achievable)

If the fluid were mineral oil, μ = 0.040 Pa·s instead of water:
$|\Delta P/L|$ = (0.0167 e-9 m^3/s) (128 / π) (0.04 Pa·s) / (50 e-6 m)^4 = 4.35 e6 Pa/m, or 131 kPa (18.7 psi) for L = 3 cm
Large increase in viscosity requires large increase in pressure to maintain flow rate

(With water): If the channel diameter is 40 μm instead of 50 μm:
$|\Delta P/L|$ = (0.0167 e-9 m^3/s) (128 / π) (0.04 Pa·s) / (40 e-6 m)^4 = 266 e3 Pa/m, or 7970 Pa (1.1 psi) for L = 3 cm
Small reduction in diameter requires noticeable increase in pressure to maintain flow rate

Note: in all of these calculations – watch the units! Easiest to convert all to SI units.

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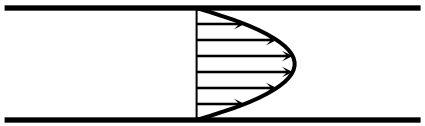
Fluid flow profiles

- Laminar flow in a channel has a characteristically parabolic flow profile
  - Velocity = 0 at channel walls (no-slip boundary condition)
  - Maximum velocity at center line



Velocity profile in 1-D flow

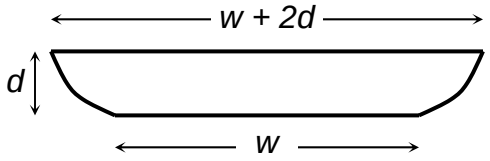
Flow between parallel plates
Spacing/depth = *H*

$$v_x(y) = \frac{H^2}{8\mu}\left|\frac{\Delta P}{L}\right|\left[1-\left(\frac{2y}{H}\right)^2\right]$$

Flow in a cylindrical tube, radius *R*

$$v_z(r) = \frac{R^2}{4\mu}\left|\frac{\Delta P}{L}\right|\left[1-\left(\frac{r}{R}\right)^2\right]$$

- In a channel of non-circular cross-section, the flow is not "parabolic", but will follow that general trend
- For wide isotropic etched channels or shallow rectangular channels



  - Flow is parabolic across the *depth* of the channel; not so much across the width (see parallel plate solution above!)
  - This is less obvious when using a typical wide-field (non-confocal) microscope to examine flow in a channel

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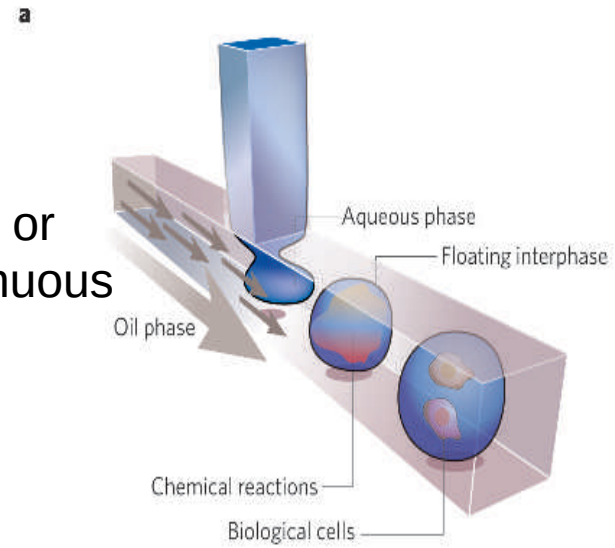
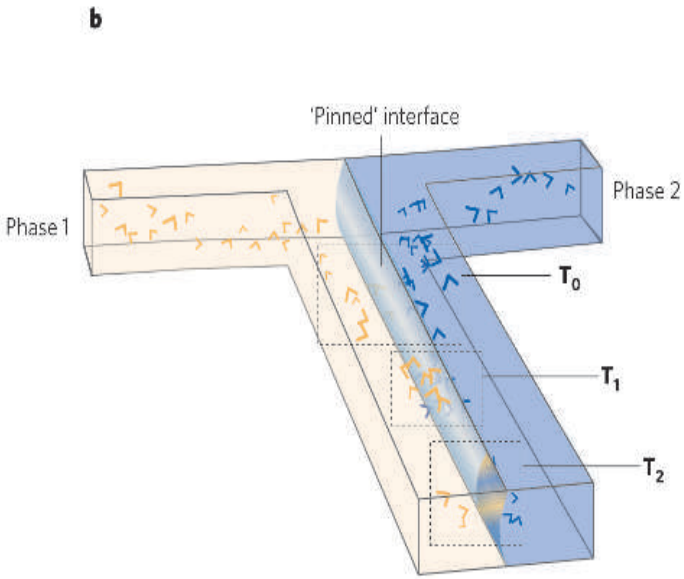
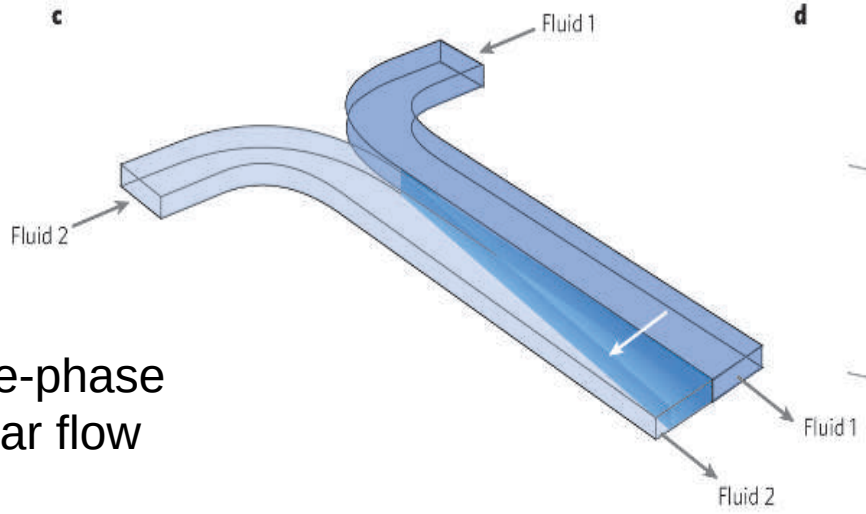
Laminar flow in microfluidics – large variety of possible behaviors

Droplets or discontinuous flow

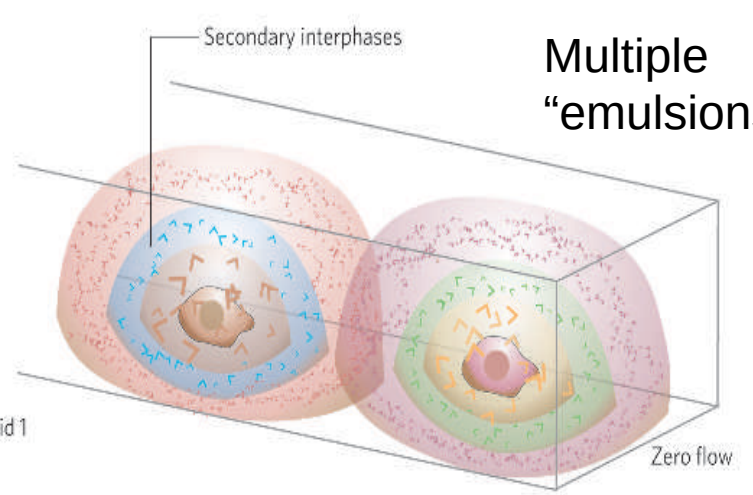




Stratified 2-phase flow



Single-phase laminar flow



Multiple "emulsions"

Atencia and Beebe, *Nature* 2005

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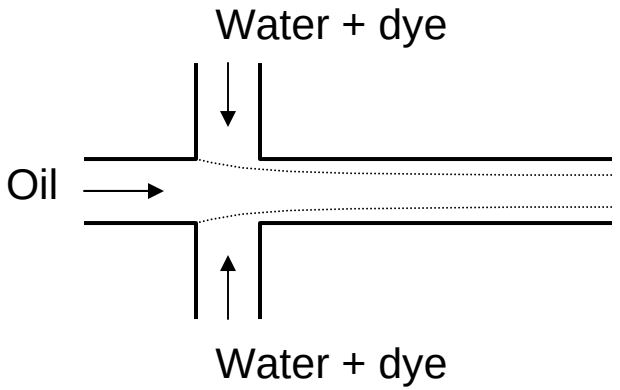
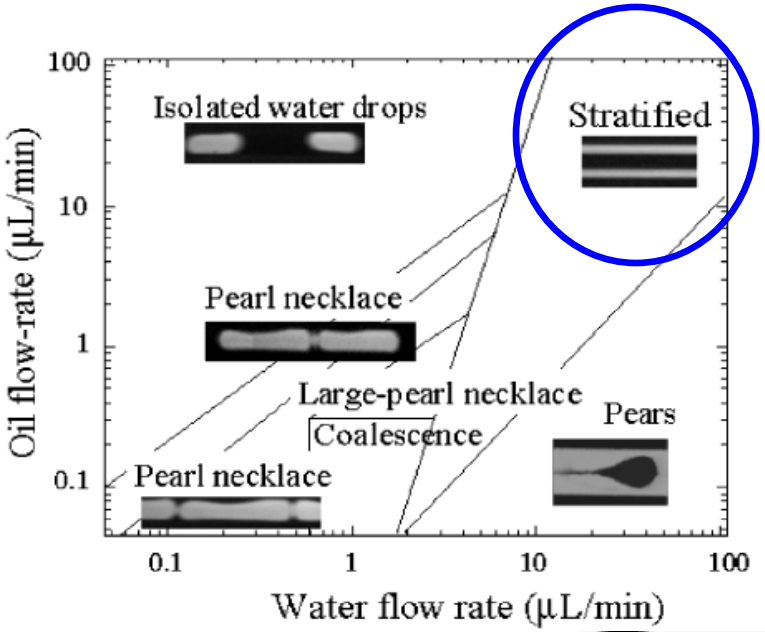
Two-phase flow in microfluidic chips

- There are numerous reports of aqueous/organic two-phase flow in microchannels.
- Buoyancy effects are negligible for microfabricated channels (1-100 µm)
- $Ca = \mu u / \gamma$ is important parameter for determining flow characteristics.
  - Small *Ca* – surface forces are dominant
  - Large *Ca* – viscous forces are dominant
- High interfacial tension in oil-water systems (~10-50 mN/m) typically leads to droplets or segregated flow (minimizing interfacial area)
- Surfactants help to achieve side-by-side or stratified flow by decreasing interfacial tension





Dreyfus *et al*, *Phys. Rev. Lett.* 2003
Hexadecane and water with Span 80

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Stratified flow

- Large *Ca* – requires high flow rates or viscosity, low surface tension
  - Oil-water systems – generally require surfactants (reduce surface tension ~ 10-fold)
  - Aqueous two-phase systems have inherently low surface tension
  - Organic two-phase systems may also have lower surface tension
  - “pinned” interfaces with patterned coatings may assist stability of interface
- Applications
  - Two-phase extraction
  - Flow patterning (*e.g.* interfacial polycondensation of nylon membrane)
  - Online sample prep/purification

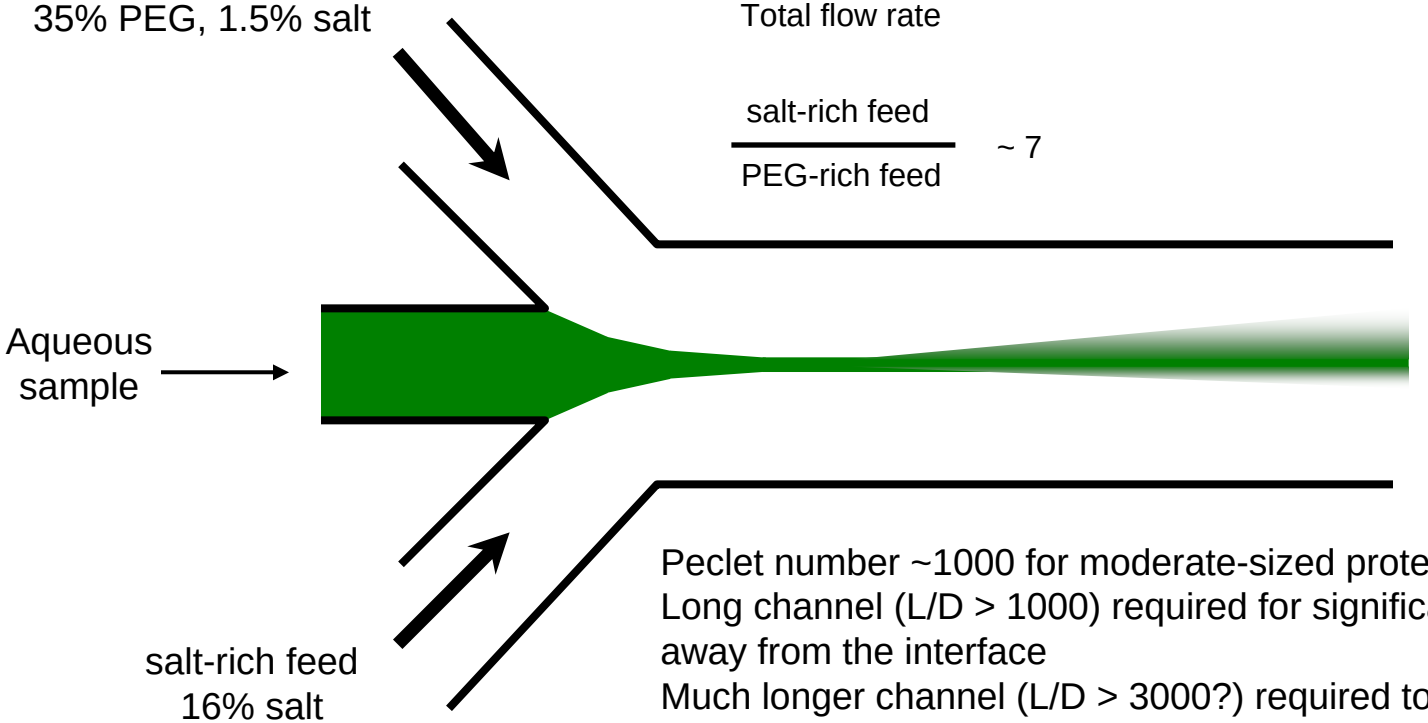
Laminar Flow Extraction (ATPS)

PEG = $HO\text{-}(CH_2CH_2O)_n\text{-}H$

PEG-rich feed
35% PEG, 1.5% salt

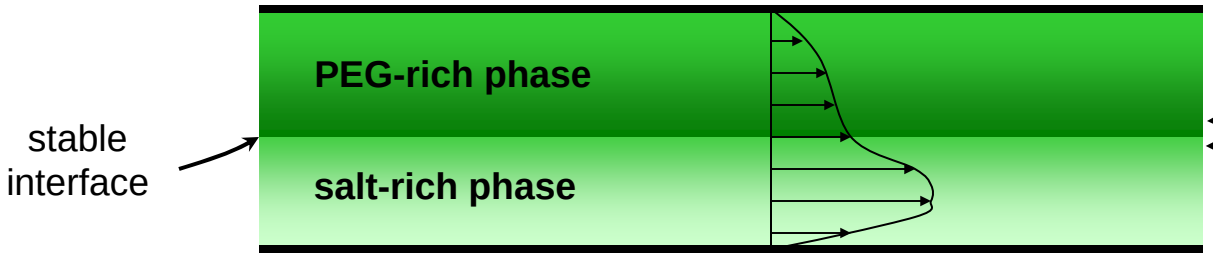
$$\frac{\text{Sample flow rate}}{\text{Total flow rate}} \sim 0.05 - 0.10$$

$$\frac{\text{salt-rich feed}}{\text{PEG-rich feed}} \sim 7$$



Peclet number ~1000 for moderate-sized protein
Long channel (L/D > 1000) required for significant sample diffusion away from the interface
Much longer channel (L/D > 3000?) required to approach 1 equilibrium stage.

Far downstream…



Equilibrium at interface

$$c_{i,PEG} = Kc_{i,salt}$$

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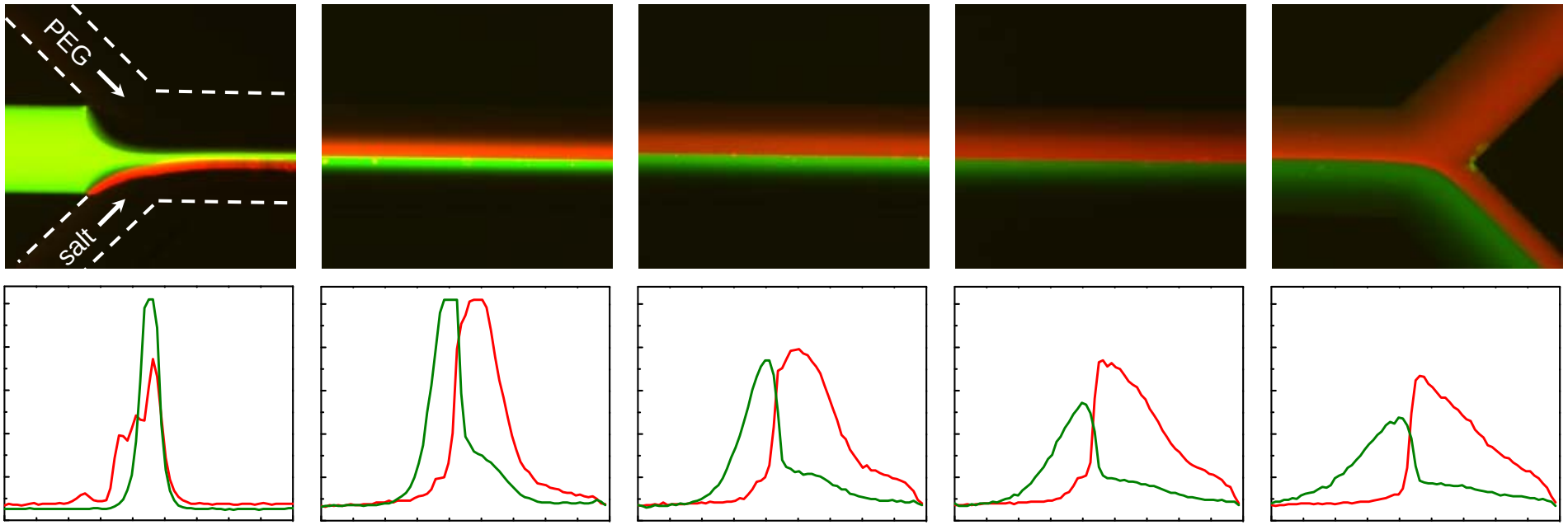
Example: fractionating proteins by two-phase extraction

- Example – fluorescently labeled BSA (green) and β-galactosidase (red) fed simultaneously
  - ΔP ~ 13.5 psi, Total flow rate ~ 7 µl/min (15 mm/s)
  - BSA partitions to salt (bottom) phase; β–gal partitions strongly to PEG (top) phase



Meagher, *Lab Chip* 2008

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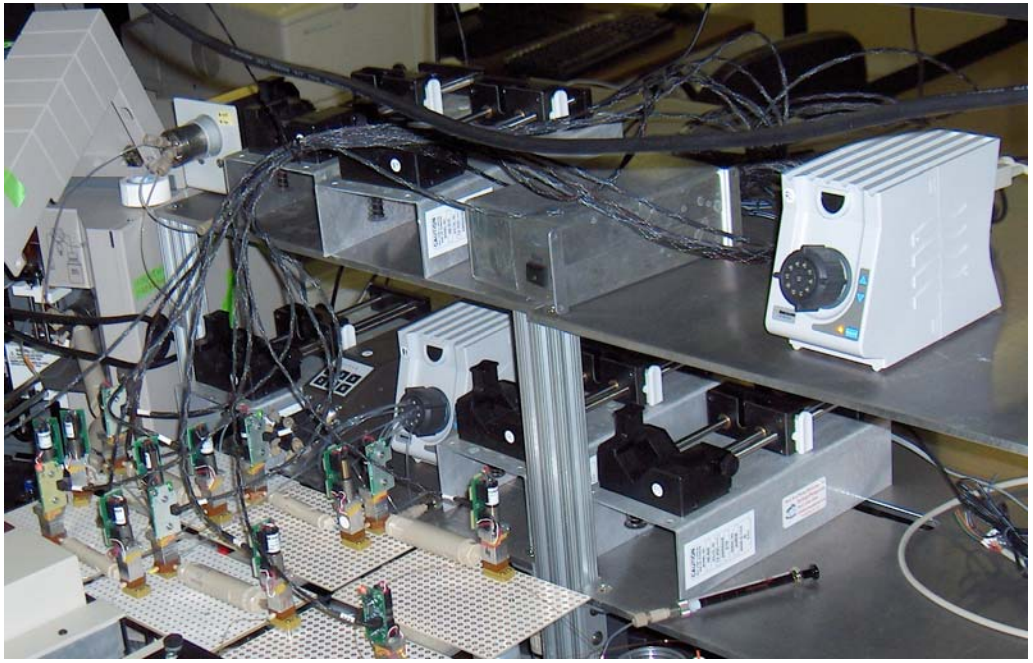
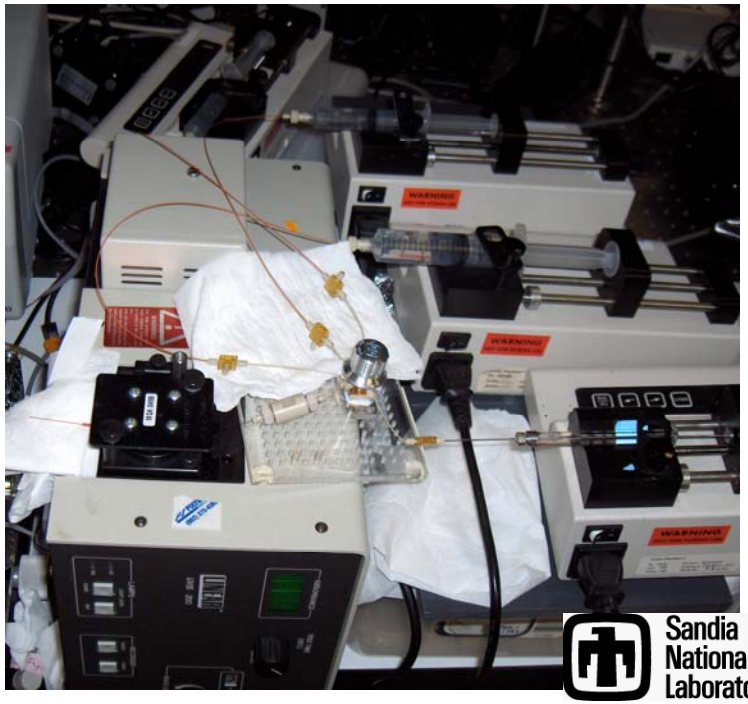
Driving flow in microfluidic devices

- Syringe pumps (~1 – 1000 µL/min)
- Pressure regulators & controllers
- Electroosmotic flow / EO pumps
- Other miniature pumps
- On-device pumping (*e.g.* PDMS valve pumps)

Syringe Pumps

- Simple, common approach
- Direct, unambiguous control over flow rate
  - Wide variety of syringe sizes provides good dynamic range
  - Pressure “floats” with flow rate (be careful, may be higher than you think)
- Typical syringe pumps are bulky (25 x 15 x 15 cm)
  - Microfluidics almost inevitably requires 2 or more pumps!
  - Complex syringe pump setups may require a large amount of real estate.





Issues with syringe pumps

- Bubbles anywhere in system (in tubing, stuck to plunger, in via holes & dead volumes, etc) significantly slow the response, especially at low plunger velocities
  - Need to wait for pressure to build or decay upon changing flow rate – can have very long time constants!
- Any other deformability in system can also damp dynamics
  - Flexible tubing
    - At 1/32” or smaller OD tubing: PEEK is less deformable than Teflon/FEP
  - Plastic syringes
  - Plastic devices
  - Deformable fittings/tubing connections (e.g. pipette-tip “fittings”)
- Stepper motor mechanism leads to pulsing at low stepping rates
  - try using a smaller diameter syringe and higher step rate
  - Reducing gears?

How to get rid of bubbles in small syringes

- Small glass syringes (<100 µL) are hard to prime with aqueous samples – bubbles stick to plunger.
- Flicking / slapping / shaking / vortexing syringe usually doesn't help.
  - Trick 1: fill syringe initially with methanol, which will wet glass and plunger easily. Push out and refill with water or sample; repeat multiple times to remove residual methanol.
  - Trick 2: fill syringe initially with an immiscible oil, low surface tension oil, and use this to push aqueous sample. Fluorinated oils (fluorinert) work nicely in glass syringes with teflon plunger, although be careful of outgassing when pulling on plunger to fill.

Interfacing syringes to tubing and devices

- Wrong way: slip needle into tight-fitting flexible tubing
  - Unsafe – if you must do this, use blunt needles
  - Poor seal, insecure
  - Bubbles may hide in Luer-lock needles
- Better #1: luer fittings with Teflon tubing attached (*e.g.* Hamilton)
- Better #2: Luer-lock to tubing adapters available from various suppliers (360 µm capillary, 1/32”, 1/16” OD; may require a tubing sleeve)

Luer adapter for syringe
(R. Renzi)

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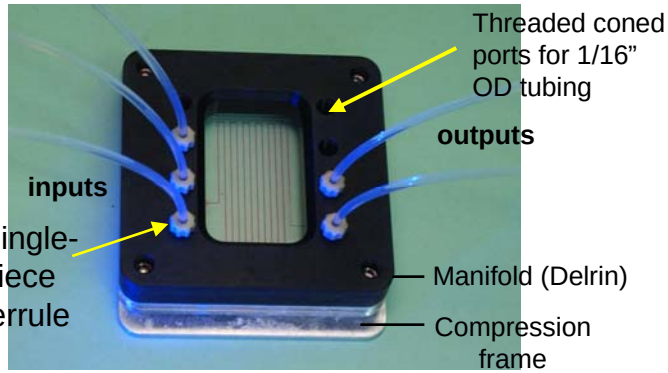
Interfacing tubing to devices

- The wrong way: jam tubing into a pipette tip, and jam the pipette tip into the device.
- Better: glued down threaded port
  - Don't clog your channels
  - may be hard to glue to some plastics
- Best: manifold with threaded ports, compression frame, O-ring seals
  - Takes some up-front engineering, and works best if you can design around a particular form factor (chip size, hole layout).
  - Some commercial versions (Micronit, Dolomite) with connections from top or edge.



Meagher *Lab Chip* 2008, Ron Renzi design, photo Huu Tran

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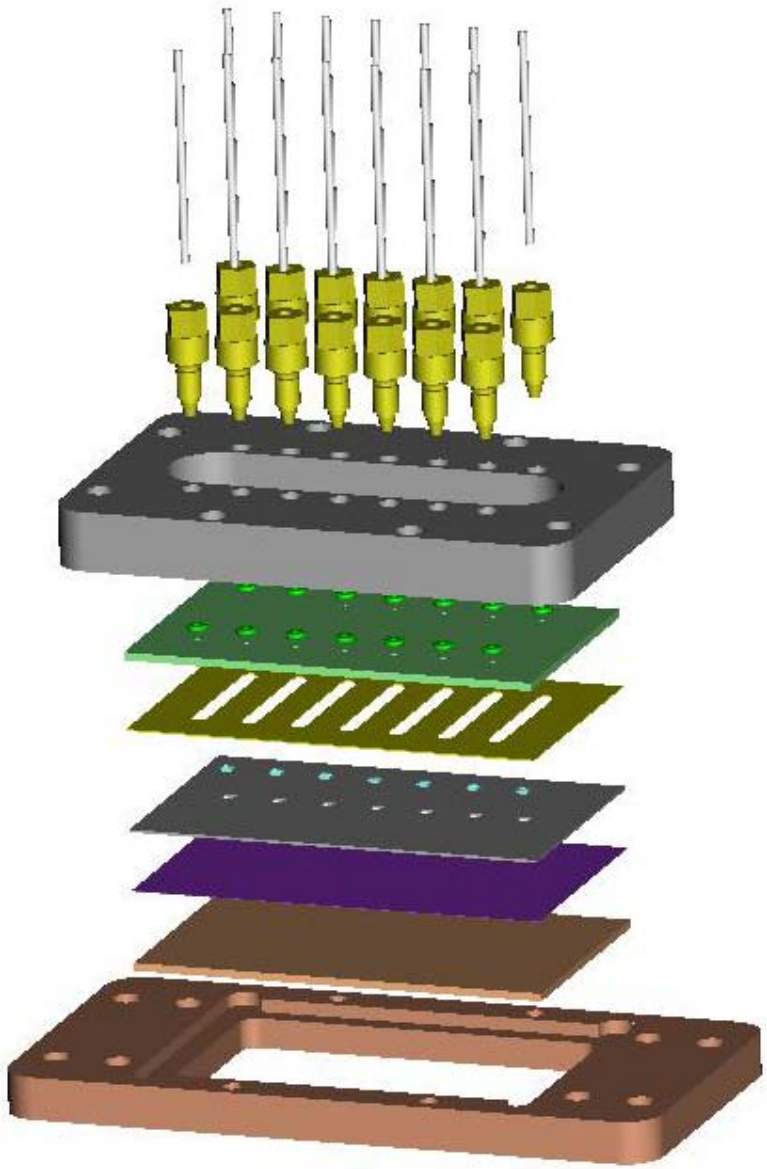

1/32” PEEK tubing

1-piece ferrules for 1/32” tubing (PEEK)

Manifold with threaded ports (Delrin) and Buna-N o-rings (not visible)

Glass chip

Additional layers…

Compression frame (aluminum)

Credit: Ron Renzi / Marc Claudnic

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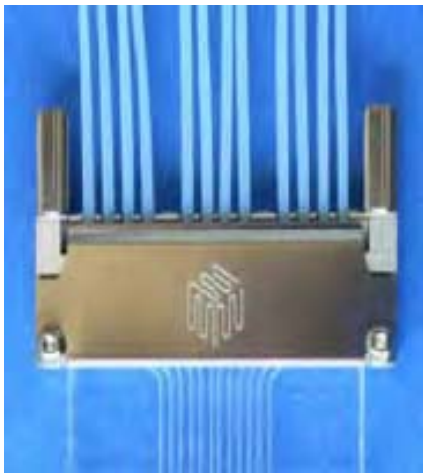
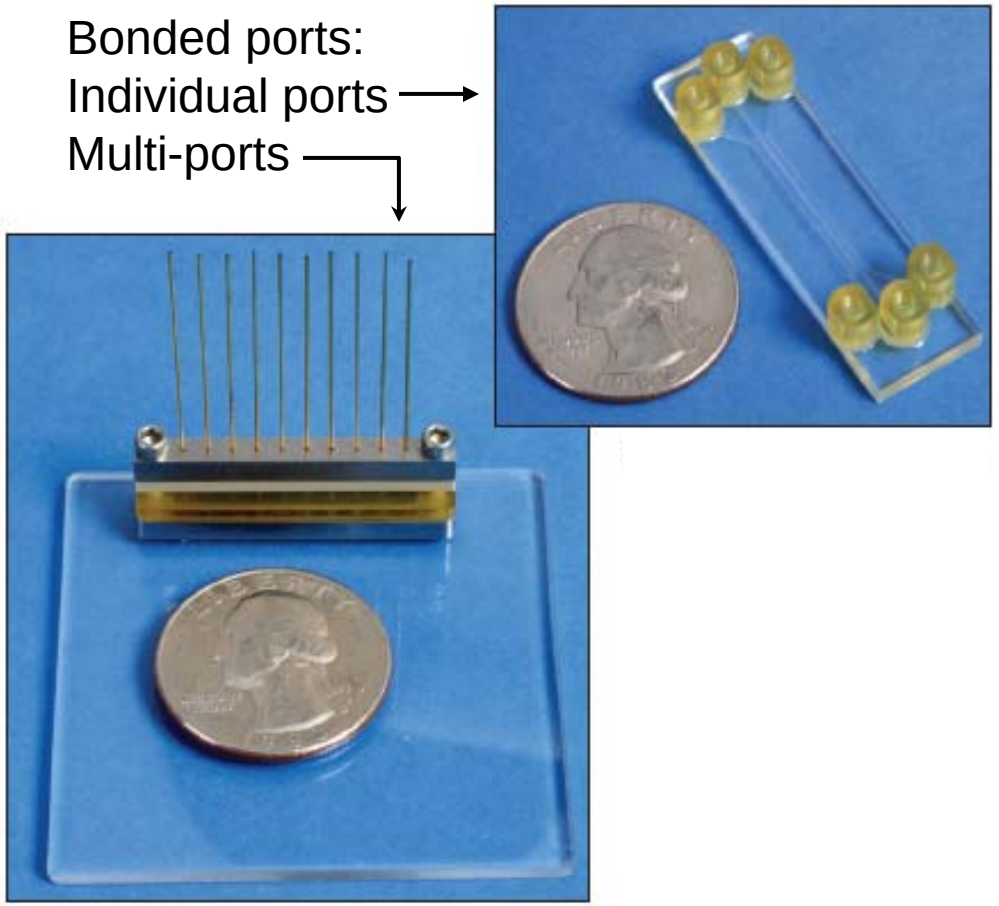


Edge connect interface
www.dolomite.com

Bonded ports:
Individual ports →
Multi-ports →



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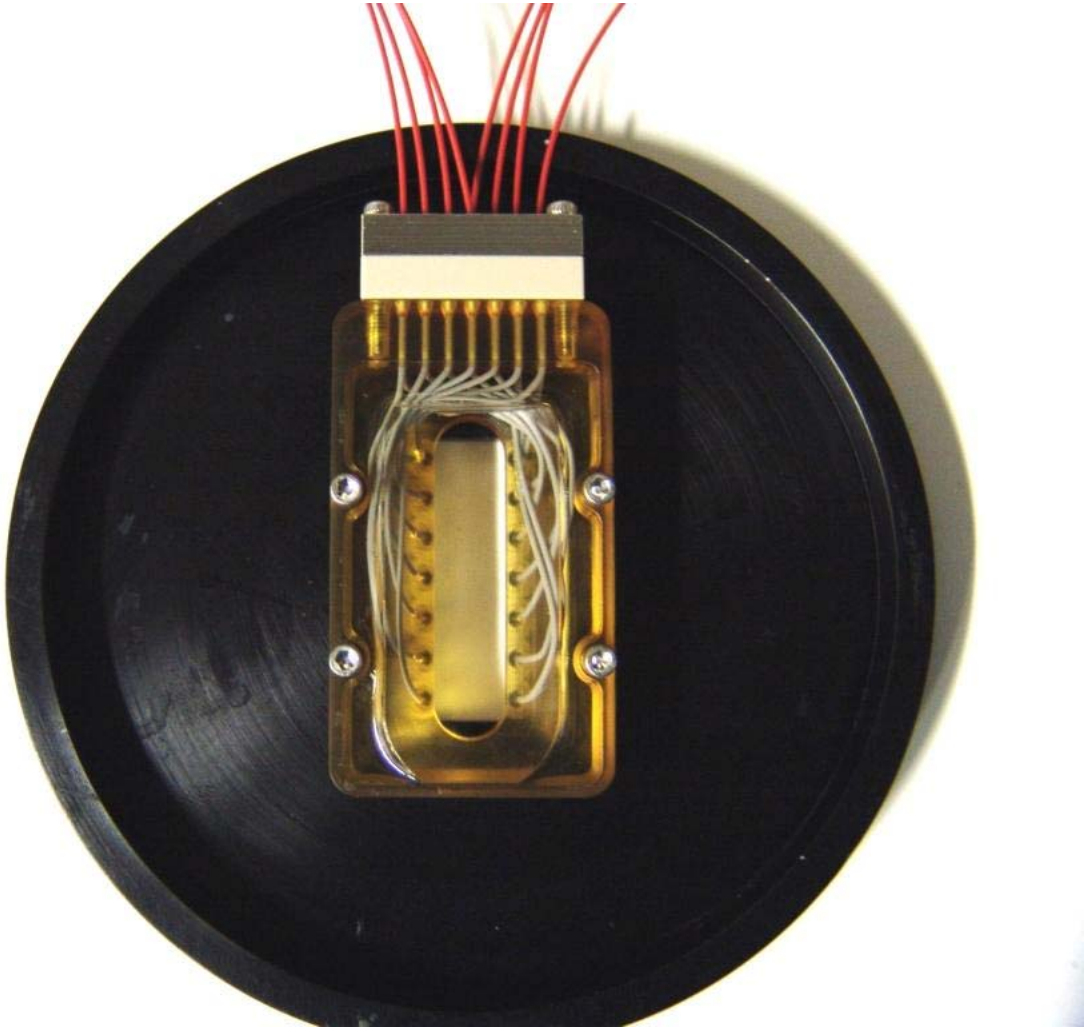
Fluidic manifold (translucent ULTEM) with edge connection interface and potted (internal) fluidic conncections to chip



Ron Renzi, Marc Claudnic, Jerry Inman

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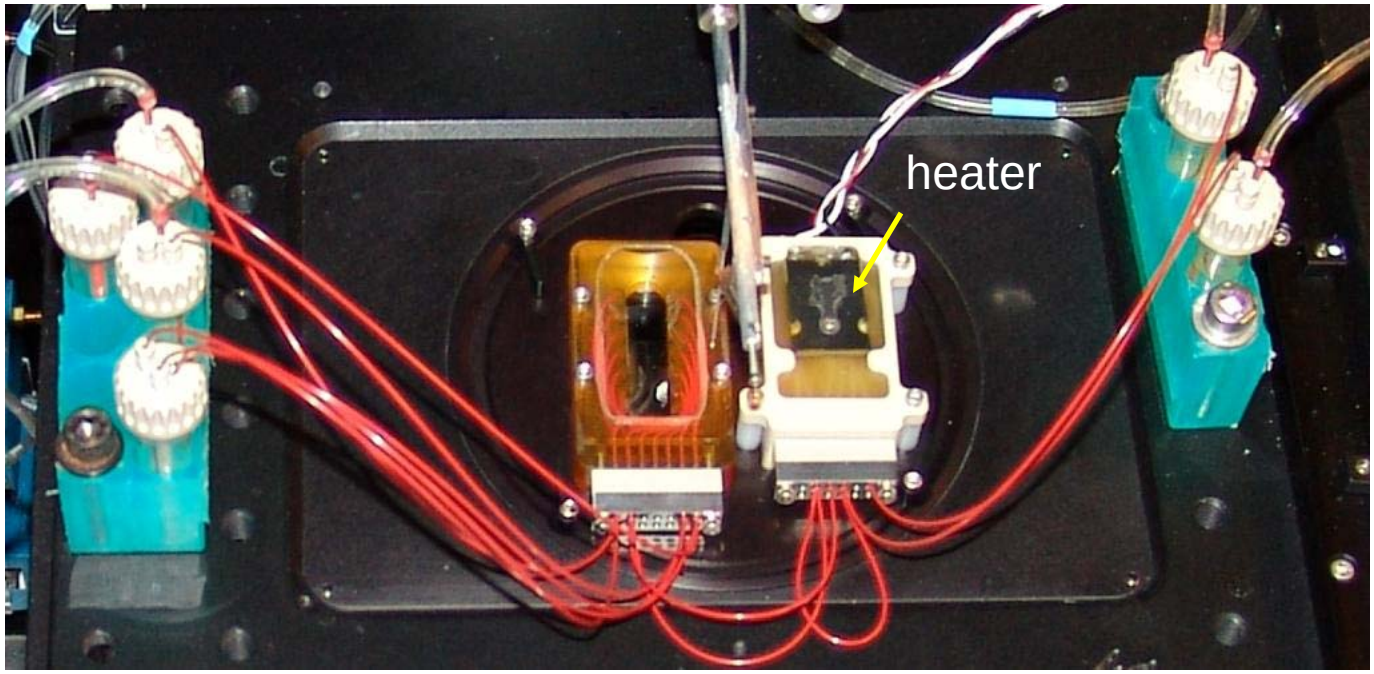

Side-by-side integrated chips in potted manifolds with edge-connect interface mounted on microscope stage-plate compression frame

Ron Renzi, Marc Claudnic, Jerry Inman, Jim Van De Vreugde

“good” materials for chip holders/fluidic manifolds

- General requirements: easy to machine, chemical resistance (appropriate to application), low autofluorescence (if applicable)
- Delrin / acetal
  - Good general-purpose material, easy to machine but somewhat soft
  - Moderately hydrophobic (θ ~ 70-80°)
  - Black delrin has low autofluorescence
- PEEK / polyether ether ketone
  - Good machinability, good chemical resistance, hydrophobic (θ ~ 90°)
  - Tends to be *highly* fluorescent (even the black version)
- ULTEM / polyether imide (PEI)
  - Strong, chemical resistant, translucent (amber)
  - Highly autofluorescent
- Kel-F / PCTFE (chlorofluoropolymer)
  - Excellent chemical resistance
  - Better mechanical properties than PTFE (Teflon)
  - Hydrophobic (θ ~ 100°)
  - Colorless translucent variety has minimal autofluorescence
- Acrylic
  - Excellent optical clarity, can be highly polished, great for making “demonstration” samples
  - Poor chemical resistance, may be autofluorescent.
- Caveat: all of these resins come in different grades, versions and flavors; your experience may vary, especially with respect to autofluorescence.

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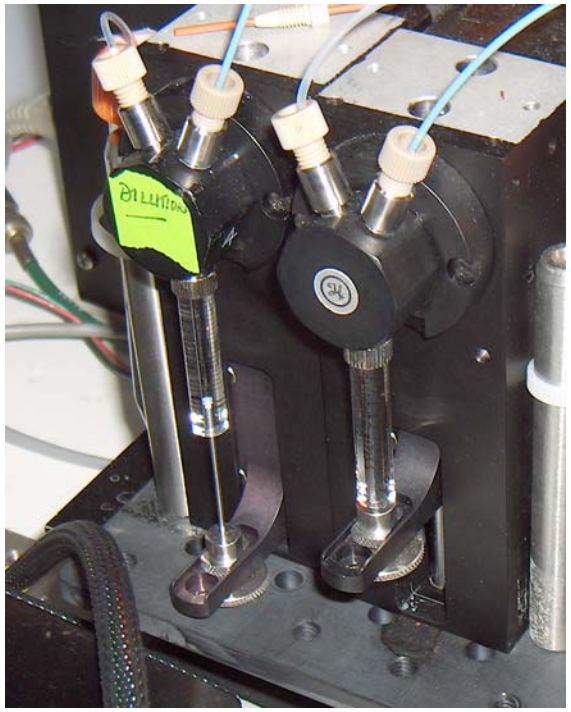
O-rings

- Buna-N, Silicone rubber tend to work well for many applications with aqueous solutions at moderate pH
- Check chemical compatibility for use with organic solvents
  - Don’t use silicone o-rings with silicone oil
  - Toluene and other aggressive solvents may require fluoroelastomers (*e.g.* Viton)
  - Many sources online for this information

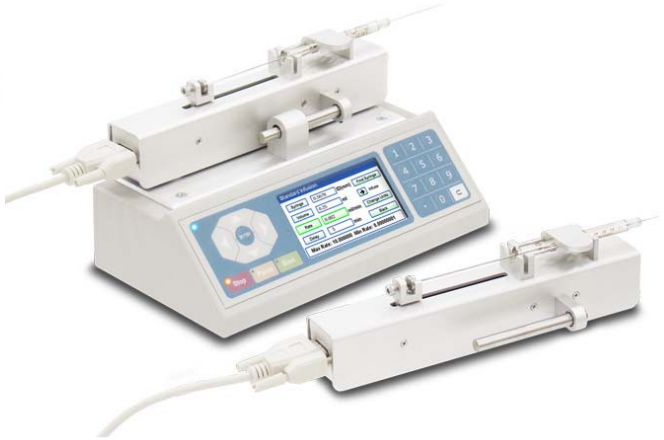
Syringe pump solutions

- To save real estate – if your process can work with fixed ratios of flow rates, try a multichannel syringe pump (ratio of syringe diameters sets flow ratio)
- Systems with a single controller and 1-4 small “satellite” pumps
- Various OEM and miniature syringe pump systems



OEM syringe pump module
External control by RS-232
No user interface, saves some space

Stereotaxic pump with controller and remote units



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Flow using pressure control

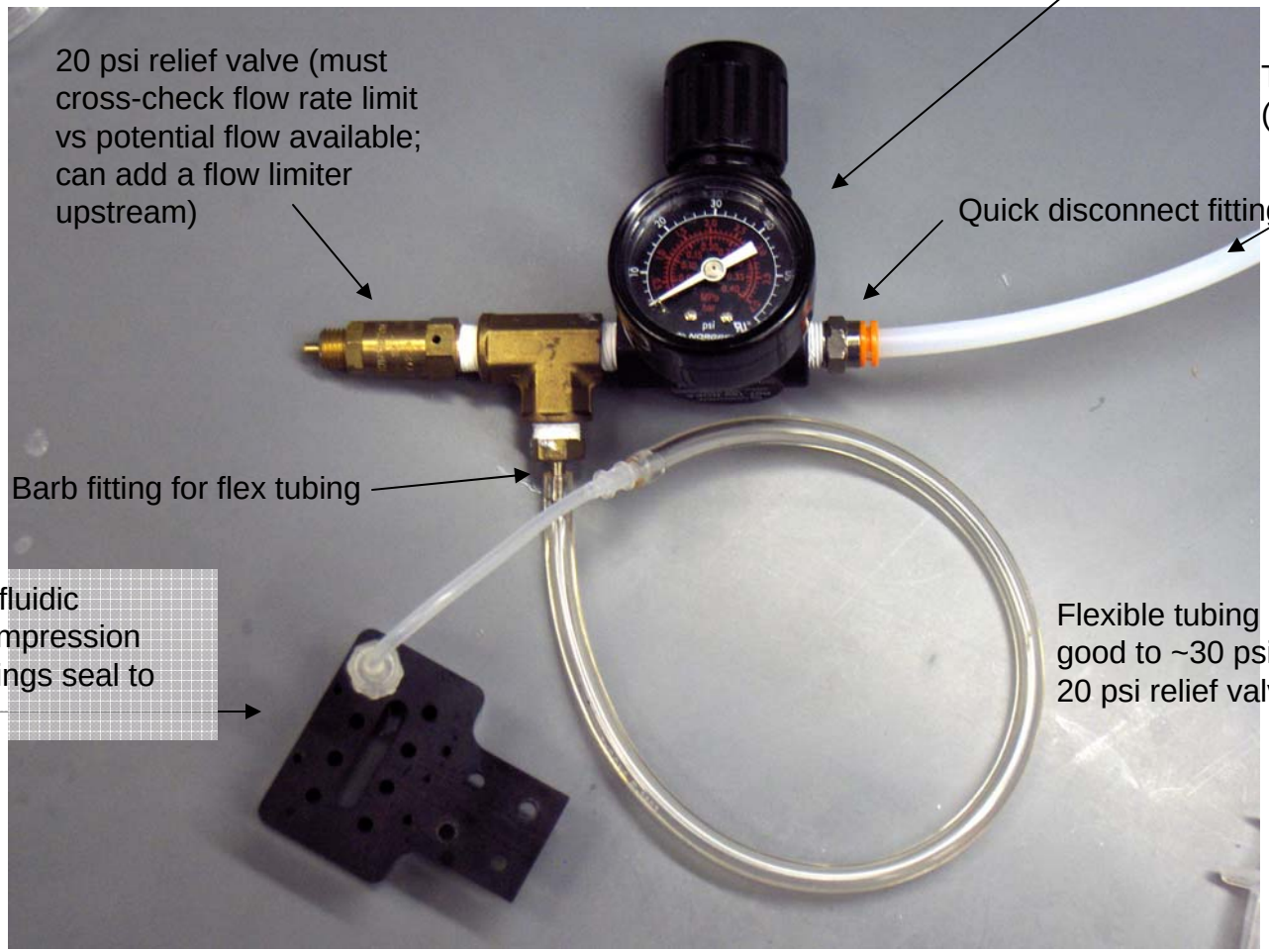
Advantages of pressure control

- Pressure changes upstream propagate quickly through the system (fluid response time is *Re*-dependent)
- Potentially simpler, less expensive, and smaller setups

Drawbacks of pressure control

- Pressure systems with a gas cylinder or high-pressure, large-volume gas source present serious but manageable safety concerns
  - requires some engineering knowledge and know-how
  - Don't let a grad student cobble something together out of luer locks and pipette tips!
- Flow rate is not controlled directly
- In complex systems with multiple pressure sources – pressures must be balanced appropriately to keep everything moving in the right direction, which is sometimes harder than it sounds.
  - Modeling of flows is helpful!
  - If possible – a single pressure (or vacuum) source to control multiple streams, with internal or external to adjust flow rate.

Simple pressure control (single stream, manual)



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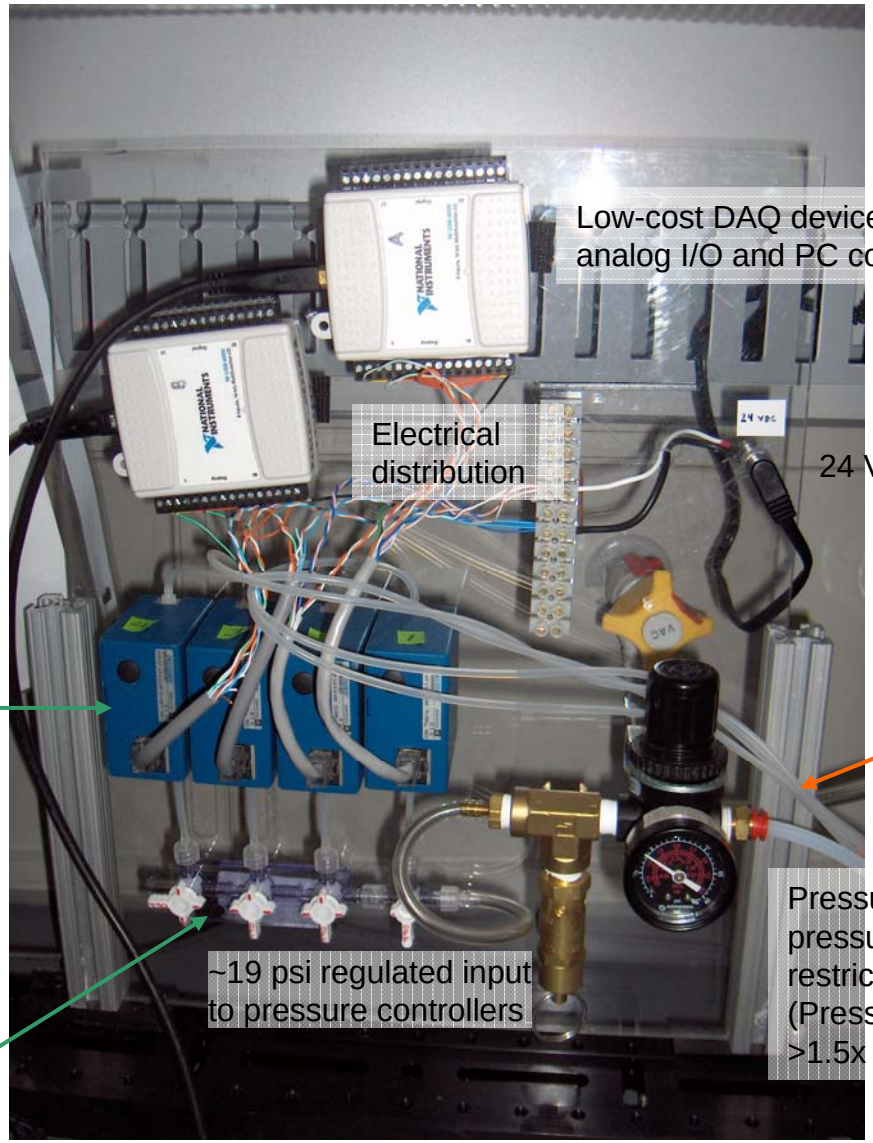
Electronic pressure control modules available from several sources, with on-board feedback control and ~10 ms response time to change in setpoint

4-channel 0-15 psi control system



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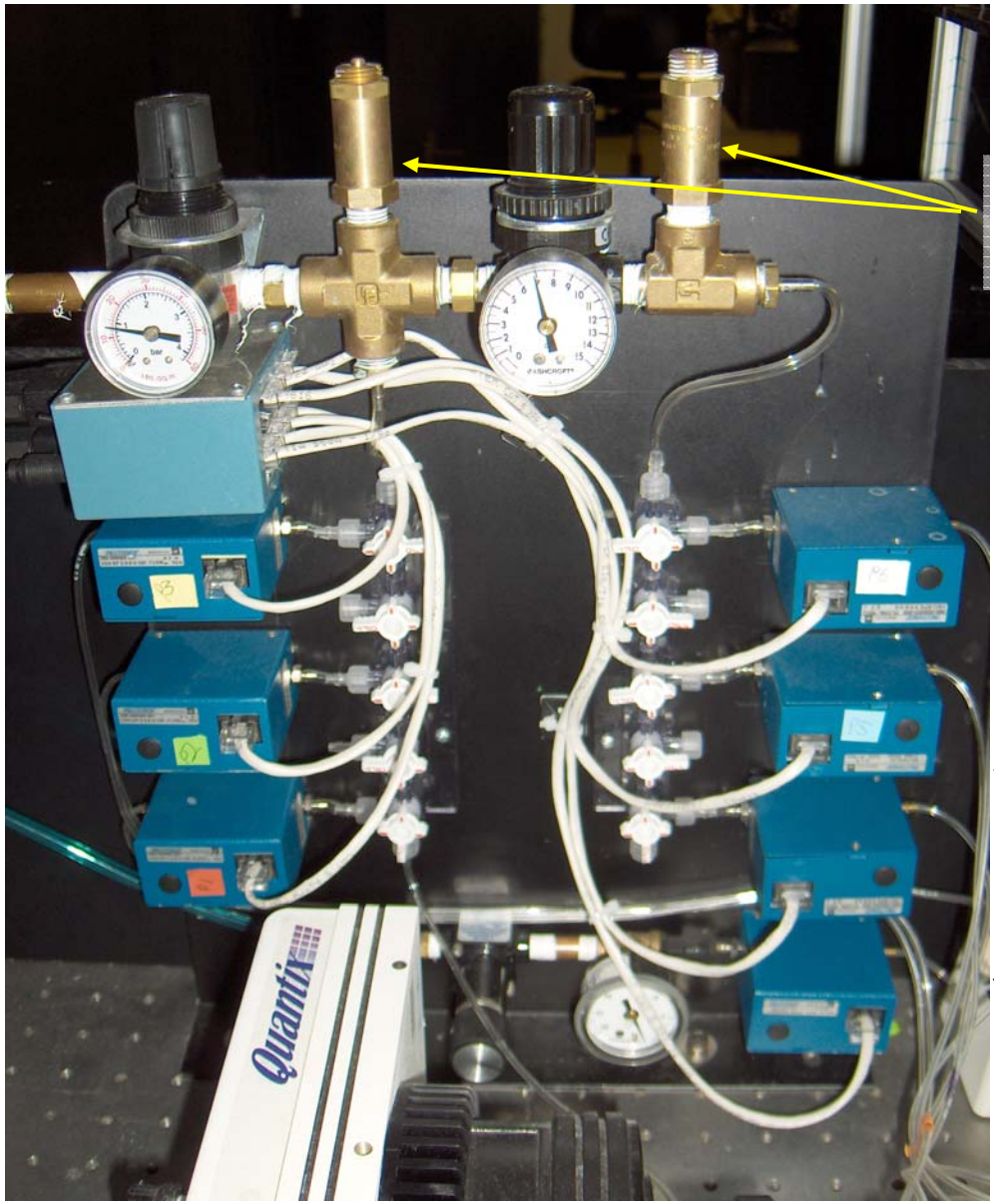
7-channel multi-range pressure/vacuum system

20 psi manifold

8 psi manifold



Remember the relief valves!

Electrical distribution box

0-5 psi controllers

0-15 psi controllers

1/32” ID tygon tubing for pressure outputs to reservoirs

Vacuum controller

K. Patel / T. Perroud, SNL

Sandia National Laboratories

Integrated benchtop chip controller package consisting of multiple-range pressure controllers, 3 independent temperature controllers, and data acquisition/signal processing hardware. Hardware designed and built by: Ron Renzi, Jim Van De Vreugde, Mark Claudnic, Jerry Inman; Funding from Sandia LDRD; Photo credit: Randy Wong

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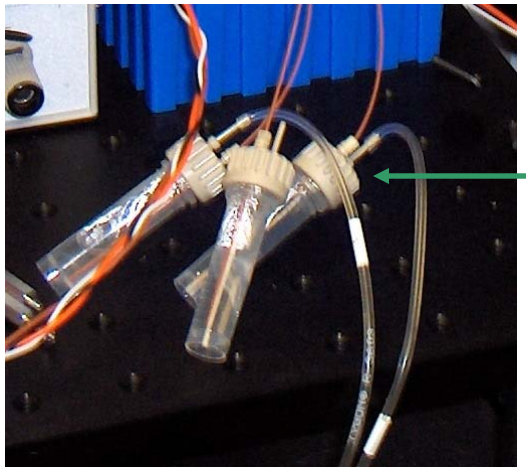
Reservoirs for reagents

- Need something (ideally small volume) to hold reagents, with 2 ports: pressure in and fluid out.
- The wrong way: septum vials (e.g. HPLC sample vials) with needles and tubing piercing septa
  - Pressurized flexible tubing terminated with sharp needles is a **bad** idea due to potential whipping hazard
- Better: pressure-rated vials or bottles with threaded ports for secure tubing connections



Custom-made 2-port cap
Fits on standard 2-mL screw top microtube
1/16” threaded port for pressure,
1/32” port for fluid flow

Electroosmotic pumps

- In principle, EO pumping is an attractive option in microfluidics – no moving parts, simple DC voltage or PWM control
- External pumps contain porous media and can be integrated with reservoirs and connected to device by tubing
- Pumps can also potentially be fabricated on device with monoliths, or native charge of walls.
- At least one commercial source for EO pumps for microfluidics
  - Didn’t pan out so well in my experience!
- Drawbacks/complications:
  - Irreproducible flow?

Monitoring flow rate on a chip

- For pressure and EO flow – it would seem attractive to monitor flow rate and perform closed-loop control.
  - Easier said than done for most of us.
  - Commercial solution?

- Various commercial flow sensors for monitoring flows in the low nL to microliter flow range, e.g. thermal anemometry
  - Standalone versions are fairly bulky by microfluidic standards, and require 1 serial connection per flow meter
  - Metering or controlling more than one flow at a time this way is tricky
- Minitaurized pressure transducers for measuring ΔP across a segment of tubing or channel with known resisitance
  - Requires some engineering and circuit design – no “plug-n-play” versions I am aware of.

Commercial nanoliter flow sensor with optional RS-232 interface to PC

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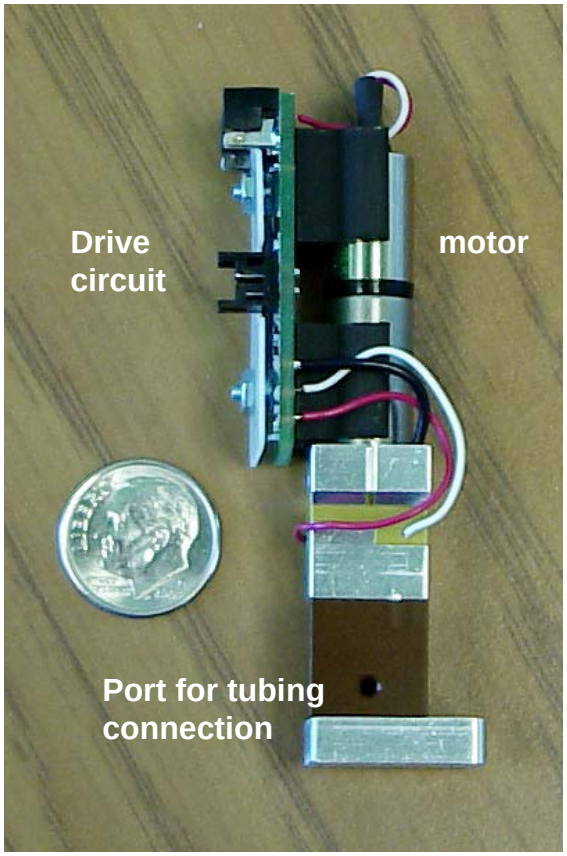
Valving

- On-chip valving
  - often some variation on a PDMS membrane being deflected by air or water pressure or other mechanical force to close off a channel
  - Versions with all PDMS (Quake), PDMS/Glass (Mathies), other.
  - Devices have at least three layers: Flow layer, membrane layer (usually a simple thin pre-fabbed PDMS sheet), and control layer (for hydraulic/pneumatic pressure)
  - Actuation of valves typically requires an off-chip solenoid to open/close air pressure to each control line
    - Solenoid actuation is typically ~10 ms, plus response time for control line/control channel
    - Moderate valving frequencies (tens of Hz) are attainable
  - Multiple variations (push-up, push-down, “leaky” valves, air-to-open, air-to-close, etc) are possible.

Off-chip valving

- In some cases, a valve can be used to block or allow flow by placing a valve on tubing off-chip
- Moves some of the complexity of fabrication off-chip (can be used with simple single-layer devices).
- Individual valves are typically more expensive, not disposable.
- Micro-valves with actuation times ranging from ~1 ms to 1 second are available
  - Often require a user-designed circuit to drive opening & closing
- Must take great care to eliminate all bubbles when connecting tubing

- With valving (on- or off-chip) – time to start/stop is a function of flow properties and system design as well as the valve actuation time
  - Very low *Re* flows ("creeping" flow) will respond very quickly to changes in pressure
  - Moderate to high *Re* flows have significant inertia and take some time to respond
  - Long control lines for on chip valves, especially with air pressure, may damp response considerably.



Renzi, Claudnic, Inman, Van De Vreugde

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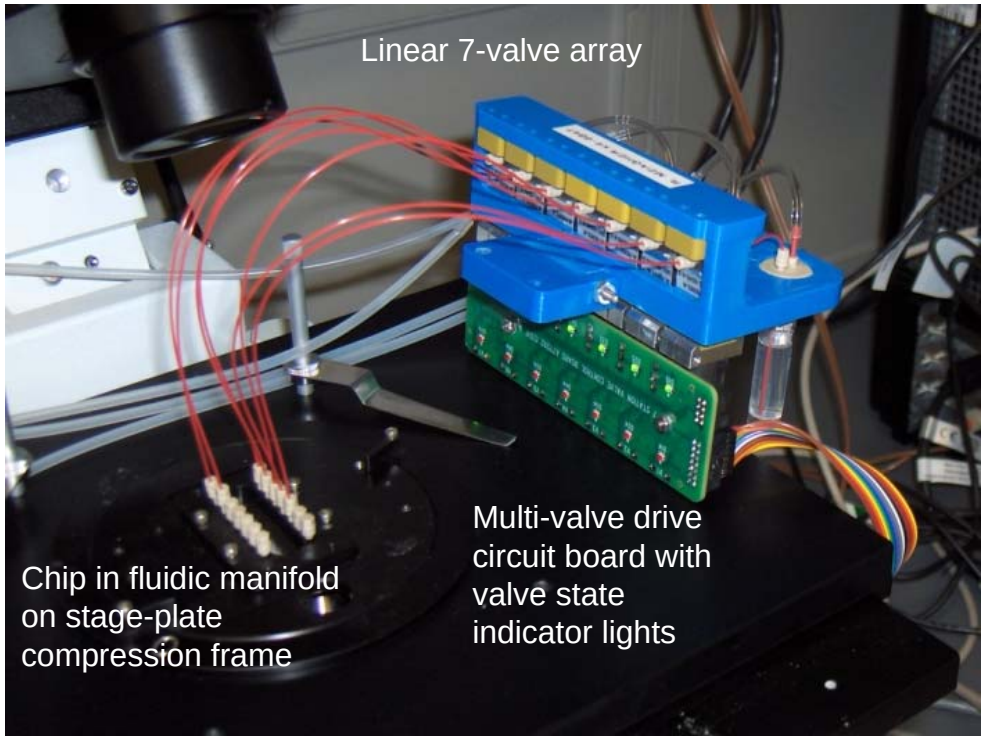
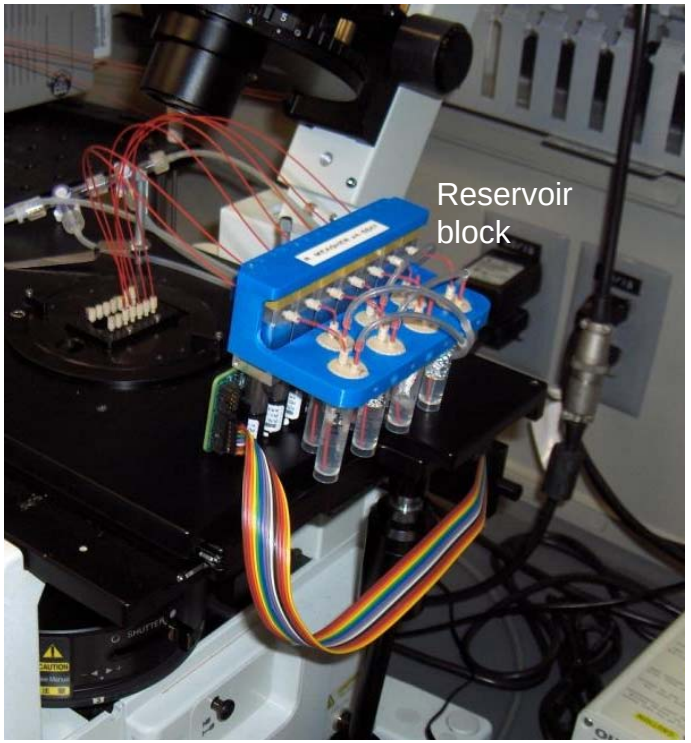
Off-chip valve block (Renzi *et al*)





Ribbon cable connects to external control circuitry for computer control, *e.g.* H-bridge array or DPDT relay board

Each valve: +9 VDC to open, - 9 VDC to close
~ 1 second actuation time

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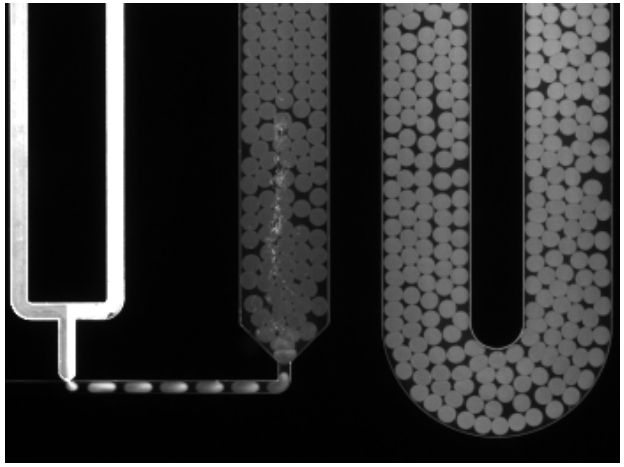
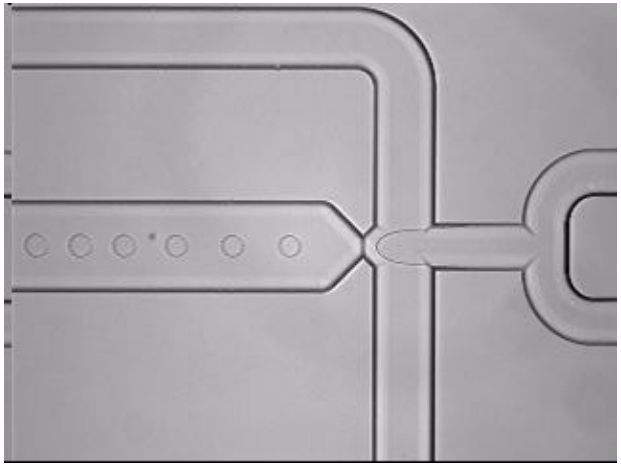
Microfluidic droplets

- Picoliter- to nanoliter- volume “emulsion” droplets in continuous flow systems
- Each droplet serves as an individual, non-communicating “test tube”
- In principle, ideal for single molecule/single cell studies
  - “Digital” PCR
  - Single-cell gene expression
  - *In vitro* transcription/translation from a single template
- Theoretically possible to perform complex manipulations / sequential operations

“T” junction



“Flow Focusing” junction



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Examples from literature

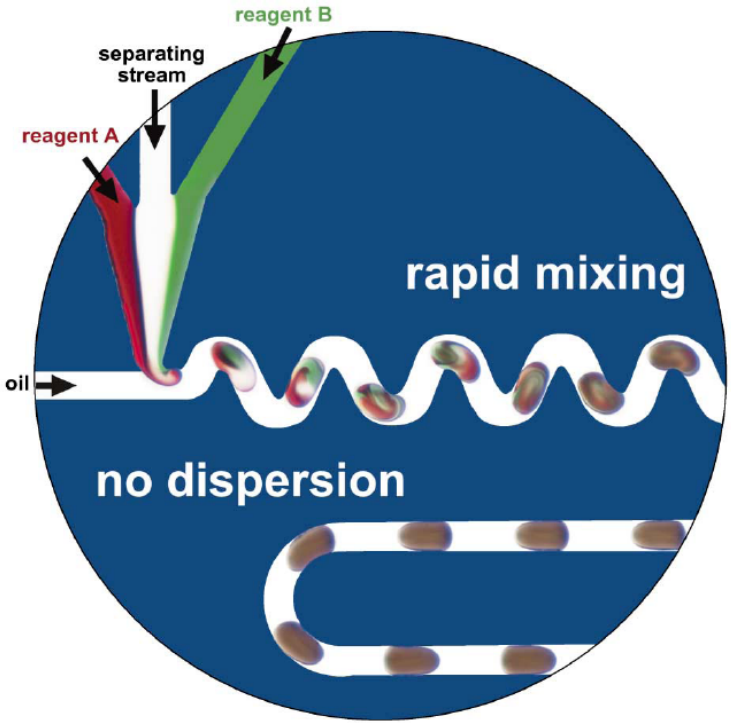
A Microfluidic System for Controlling Reaction Networks in Time**

*Helen Song, Joshua D. Tice, and Rustem F. Ismagilov**

Angew. Chemie 2003

Angewandte Chemie

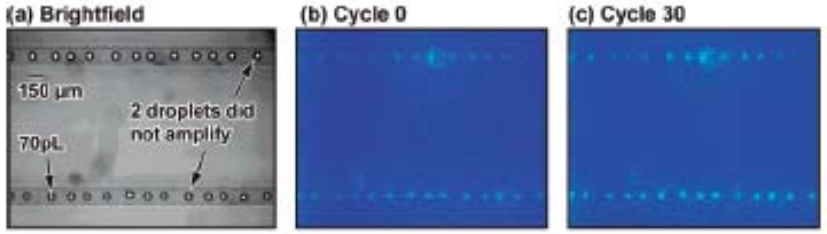
Communications



On-Chip Single-Copy Real-Time Reverse-Transcription PCR in Isolated Picoliter Droplets

N. Reginald Beer,*,† Elizabeth K. Wheeler,† Lorenna Lee-Houghton,‡ Nicholas Watkins,§ Shanavaz Nasarabadi,† Nicole Hebert,† Patrick Leung,† Don W. Arnold,|| Christopher G. Bailey,† and Bill W. Colston†

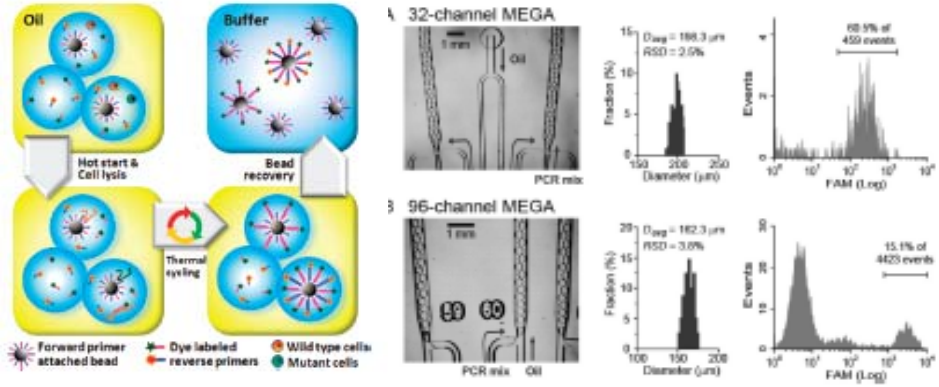
Anal. Chem. 2008



High-Performance Single Cell Genetic Analysis Using Microfluidic Emulsion Generator Arrays

Yong Zeng,† Richard Novak,‡ Joe Shuga,§ Martyn T. Smith,§ and Richard A. Mathies*,†

Anal. Chem. 2010



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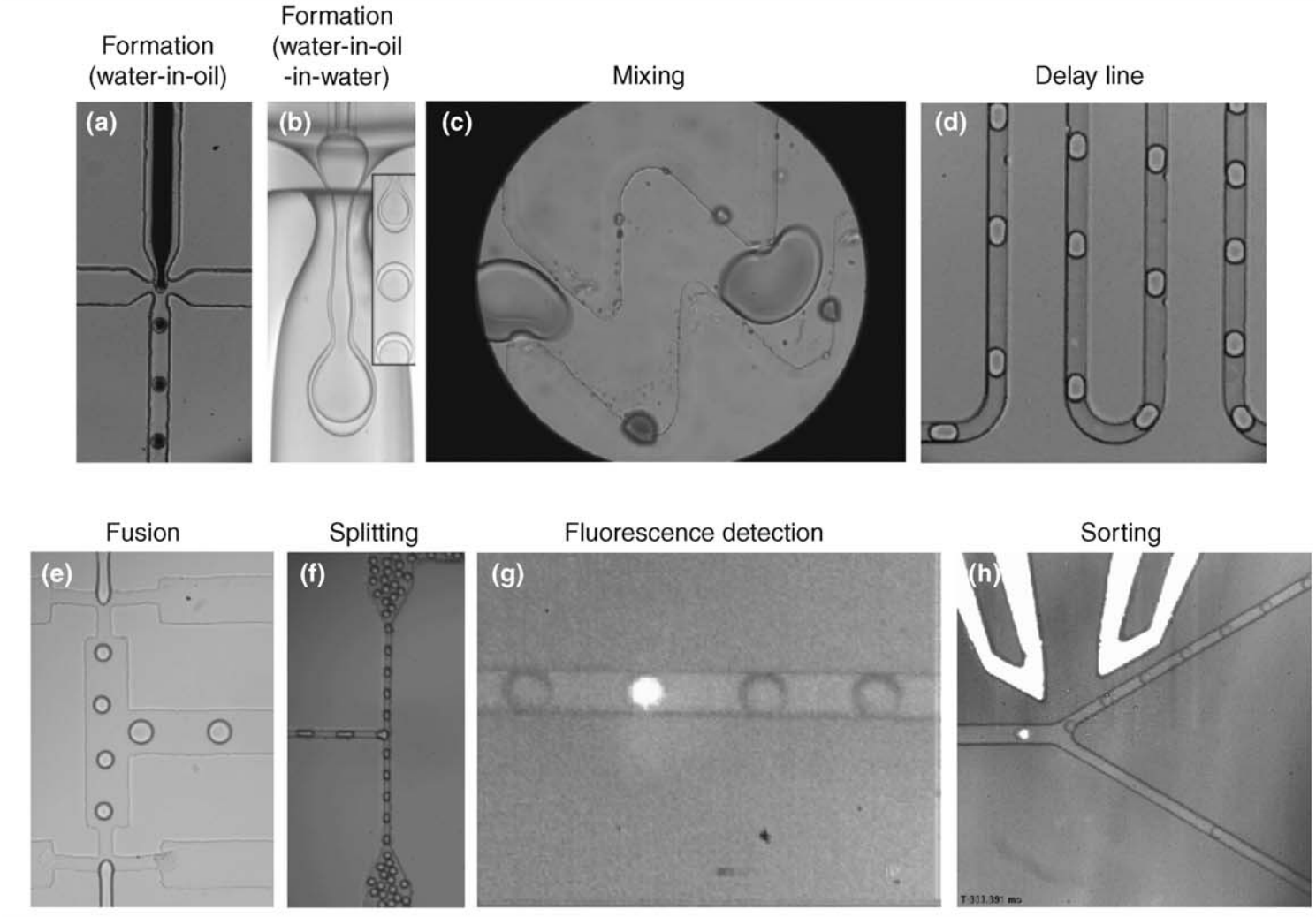
Miniaturising the laboratory in emulsion droplets

Andrew D. Griffiths[1] and Dan S. Tawfik[2]

Trends Biotechnol. 2006



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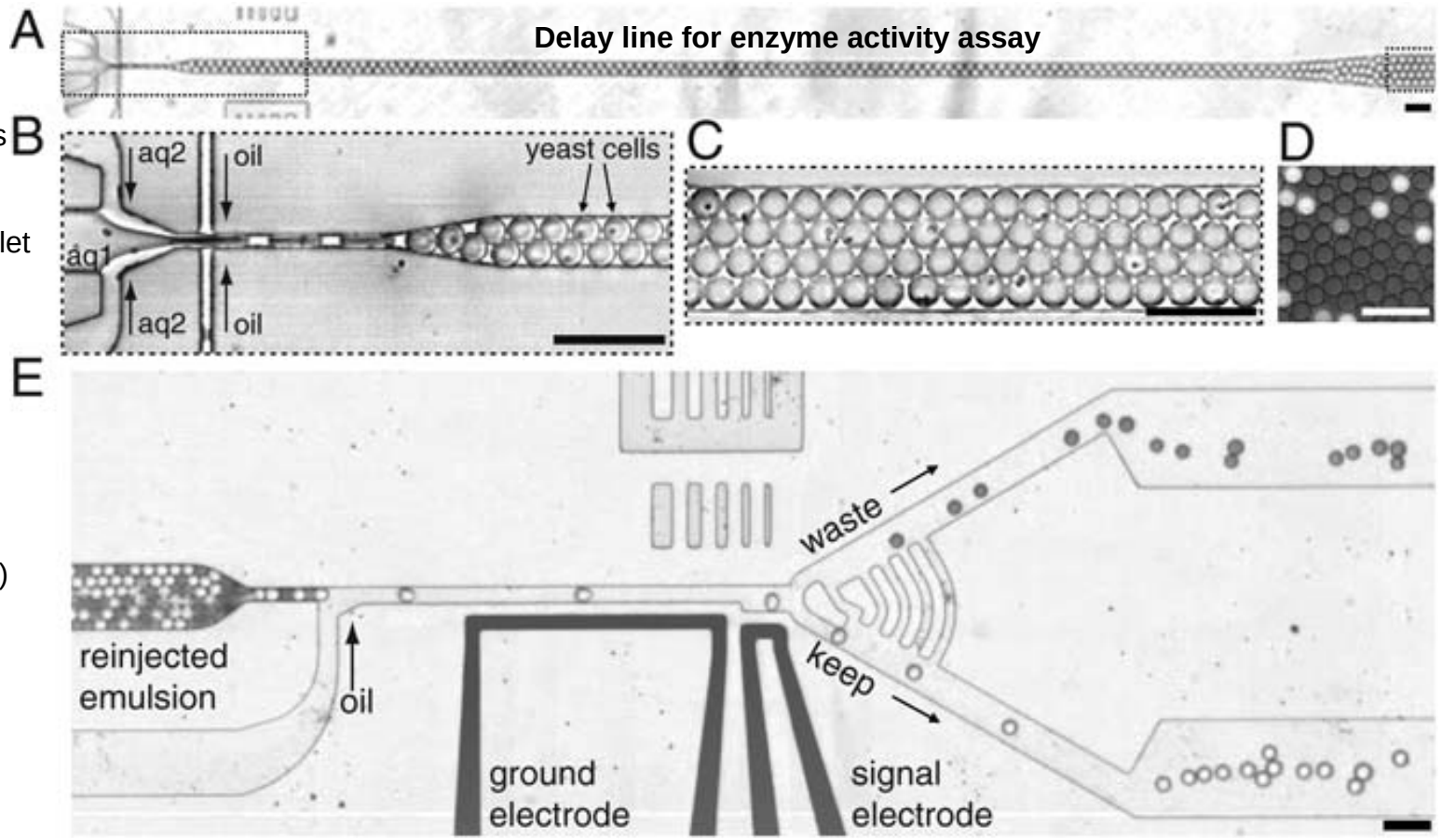
Ultrahigh-throughput screening in drop-based microfluidics for directed evolution

Jeremy J. Agresti[a,b,1], Eugene Antipov[c], Adam R. Abate[a], Keunho Ahn[a], Amy C. Rowat[a,d], Jean-Christophe Baret[e], Manuel Marquez[f], Alexander M. Klibanov[c,g], Andrew D. Griffiths[e], and David A. Weitz[a,d]

PNAS 2010



Mixing yeast cells and substrate immediately upstream of droplet generator

Fluorescence-activated droplet sorting (electrical)

The hard part happens off-chip… (library generation, mutagenesis, etc.)

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Agresti *et al*, *PNAS* 2010

Table 1. Comparison of time and costs* for the complete screen using traditional methods and in microfluidic emulsions

	Robot	Microfluidic drops
Total reactions	5×10^7	5×10^7
Reaction volume	100 μL	6 pL
Total volume	5,000 L	150 μL
Reactions/day	73,000	1×10^8
Total time	~2 years	~7 h
Number of plates/devices	260,000	2
Cost of plates/devices	$520,000	$1.00
Cost of tips	$10 million	$0.30
Amortized cost of instruments	$280,000	$1.70
Substrate	$4.75 million	$0.25
Total cost	$15.81 million	$2.50

*Details in *SI Text*

Chem Biol 2005

Chemistry & Biology, Vol. 12, 1281–1289, December, 2005, ©2005 Elsevier Ltd All rights reserved. DOI 10.1016/j.chembiol.2005.09.012

High-Throughput Screening of Enzyme Libraries: Thiolactonases Evolved by Fluorescence-Activated Sorting of Single Cells in Emulsion Compartments

Amir Aharoni,[1,3] Gil Amitai,[1] Kalia Bernath,[1,2] Shlomo Magdassi,[2] and Dan S. Tawfik[1,*]

High-Throughput Screening of Enzyme Libraries: In Vitro Evolution of a β-Galactosidase by Fluorescence-Activated Sorting of Double Emulsions

Enrico Mastrobattista,[1,3,4] Valerie Taly,[1,2,3,5] Estelle Chanudet,[1] Patrick Treacy,[1,2,5] Bernard T. Kelly,[1] and Andrew D. Griffiths[1,2,5,*]

Similar assays done using conventional emulsions and FACS
Probably higher throughput is possible this way
lacks uniformity of droplet size, but may be possible to correct for using FSC and custom sort gates

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How to generate droplets on a chip

In the simplest case, the T-junction and flow-focusing junction are basically “passive” devices – just flow the aqueous and oil streams, and fluid mechanics does the work for you.

Stable droplet production requires stable flow control!

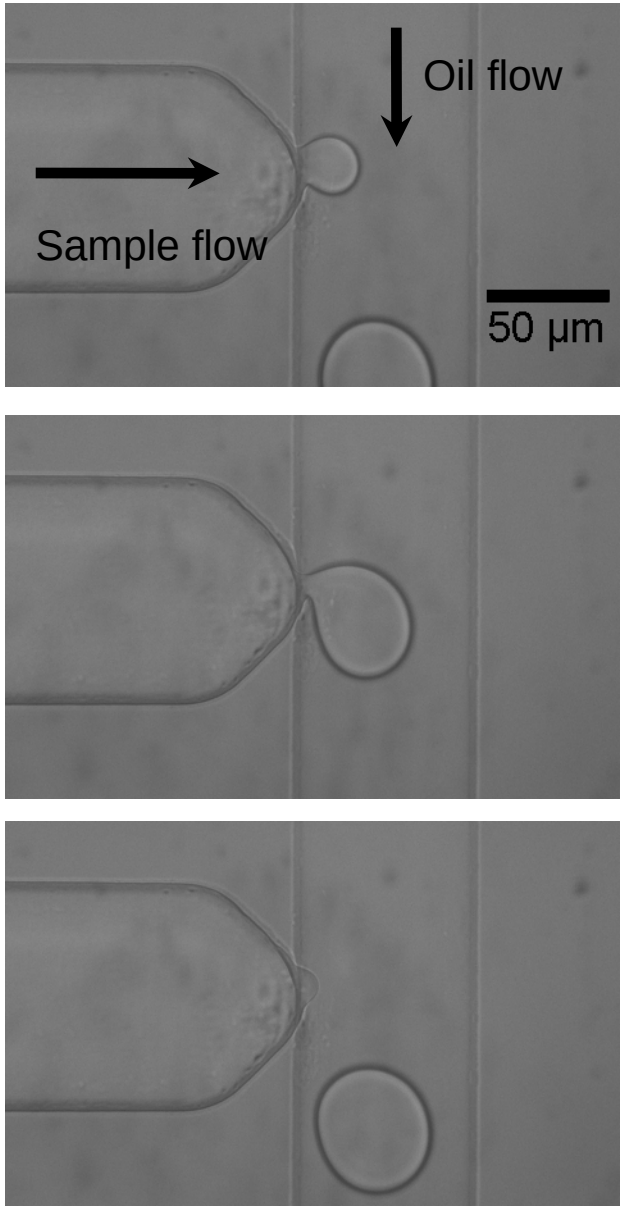
PDMS seems to be popular, but glass works too.

Surface chemistry is important: for water-in-oil droplets, the junction and oil flow channels must be **hydrophobic**.

PDMS – natively hydrophobic, but you may want to coat it anyway.

Glass – needs to be coated for w/o droplets.

If you want your droplets to be stable, you need **surfactants.**



Perroud, Meagher, *et al* *Lab Chip* 2009

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How to make glass hydrophobic

- Type of coating depends on your oil phase
  - Hydrocarbons, mineral oils, silicone oils, vegetable oils, etc – need a “conventional” hydrophobic coating
  - Fluorinated oils – need a fluorinated (Teflon-like) “superhydrophobic” coating

- Easy way: commercial window glass treatments
  - Rain-X: Silicone wax dispersed in a solvent?
  - Aquapel: reactive fluoropolymer, reportedly works on PDMS

- Silane coatings: slightly harder, but more precise (?)
  - Hydrophobic coating: octadecyltrichlorosilane, 1-2% v/v in hexadecane, toluene, or other dry solvent
  - Fluorinated coating:(heptadecafluro-1,1,2,2-tetrahydrododecyl)trichlorosilane, 1-2% v/v in hexadecane works well enough.

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Silane coatings for hydrophobic glass channels

- Literature on silane treatments is full of mysterious, sometimes contradictory protocols.
  - Much of the difficulty (and confusion) is centered around getting the perfect, smooth, self-assembled monolayer
- For the sake of simply making glass hydrophobic, **less care is necessary**.
- For trichlorosilanes – basically deposit on clean glass from a dry, aprotic solvent
  - don't go nuts getting rid of the water though, no need for distilled solvents, molecular sieves, glove box, etc. You just want to avoid "bulk" water which will cause the silane to polymerize.
  - Silane reacts with adsorbed water on glass – don't flame-dry your chip…
  - Keep your silanes in a dessicator; dispose after 1-2 years when too much crud builds up on bottle.
- Trimethoxysilanes are less reactive than trichlorosilanes
  - More stable in storage, but still keep in a dessicator
  - Wider variety of reaction conditions reported
  - Can be deposited from dry aprotic solvents or from alcohols, sometimes with a touch of weak acid (*e.g.* acetic acid) or base (*e.g.* TEA) and a bit of water added
  - Usually a longer coating time (30-60 minutes), heat cure may help

For detailed technical information on silane chemistry and silane coatings: www.gelest.com (also a supplier of many silane reagents)

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Basic protocol for octadecyltrichlorosilane (OTS) coating

1. Make sure your chip is “clean” (if you’re not sure, some combination of 1M NaOH, 1M HCl, methanol. For a new chip I usually don’t bother doing anything)
2. Flush chip for 2 minutes with methanol (to remove water)
3. Flush chip for 2 minutes with isopropanol (to remove methanol)
4. Flush chip for 2 minutes with hexadecane (or whatever dry solvent you are using for the silane) – point is to get rid of residual alcohol.
5. Flush chip for 5-10 minutes with 2% (v/v) OTS in hexadecane
6. Flush chip with hexadecane (no OTS) for 2 minutes [critical step!]
7. Flush chip with isopropanol
8. Flush chip with methanol
9. Dry with nitrogen or vacuum, or use directly. Heating at 110-120 °C for 15-20 minutes may help “cure” the coating, but in my experience this is unnecessary.

Basic protocol for heptadecaflurododecyltrichlorosilane coating is similar

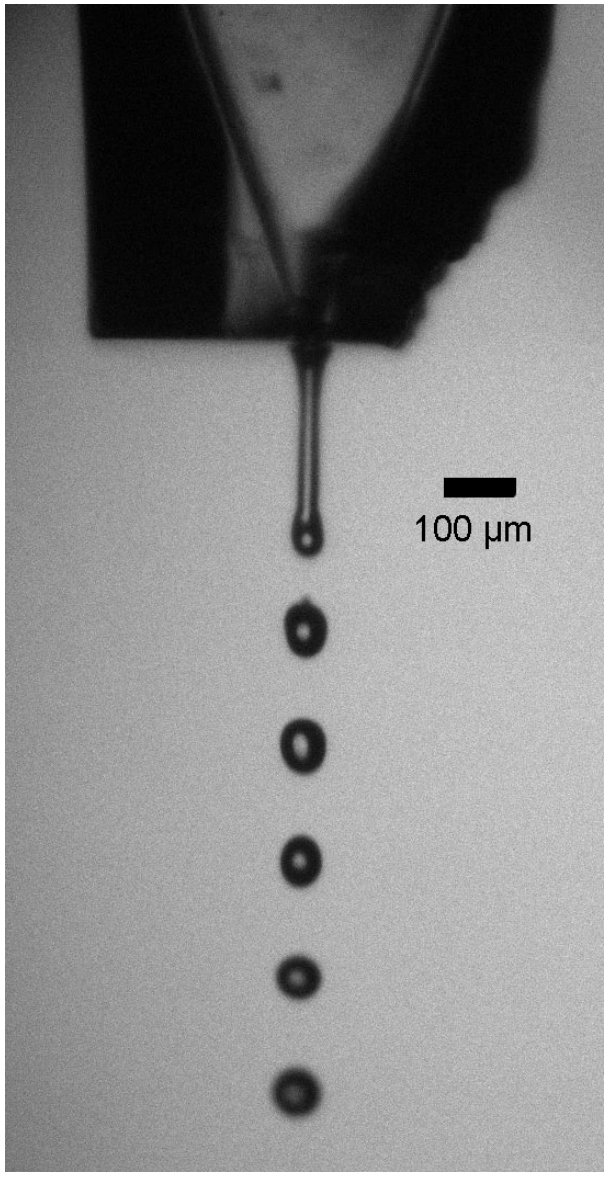
1. Hexadecane works as a solvent for this; not sure about toluene or others; could probably use a fluorinated solvent (e.g. Fluorinert) as well.
2. This silane polymerizes rapidly from moisture in air – quickly forms a “skin” over exposed surfaces. Make sure you keep this “skin” from forming over the entrance to your channel (e.g. keep a large “puddle” of the coating solution over your via hole.
3. The resulting coating is both hydrophobic and oleophobic (both water and organic solvents will “bead up” nicely on this surface).

Oil phases for on-chip w/o emulsion formation

- Mineral oil/hydrocarbon oils
  - Mineral oil + 2-5% SPAN 80
  - Mineral oil + 4.5% SPAN 80, 0.4% Tween 80, 0.05% Triton X-100 (“classic” emulsion PCR surfactant mixture)
  - Mineral oil or hexadecane + 2-4% Abil EM 90
  - Tegosoft DEC + Mineral Oil + Abil WE09 (very stable w/o emulsions, works for emulsion PCR, but aggressive to some plastics)
  - Many other possibilities (SPAN 80 and SPAN 85 are common surfactants)
- Silicone oils
  - Silicone surfactants available from Dow Corning (and others?)
  - 60/40 (v/v) PDMS oil (20 cSt) / DC Formulation Aid 5225C – stable droplets
  - 40/30/30 (v/v/v) DC 5225C / DC 749 fluid / AR20 oil
    - another classic emulsion PCR mixture (Margulies *Nature* 2005), viscous
    - Papers from Mathies group: “microfines” in emulsion enhance stability at high temperature
- Fluorinated oils (typically very low partitioning of non-fluorinated molecules)
  - Perfluorodecalin
  - Fluorinerts (FC-40 and FC-70 are higher boiling; FC-70 is higher viscosity)
  - Raindance Technologies has proprietary non-ionic fluorosurfactant, may be available through partnership/MTA
    - Gives very stable emulsions!
  - Other fluorosurfactants (*e.g.* Krytox, DuPont), mostly ionic
  - Fluorinerts tend to outgas at high temperatures

Do you need a chip to make droplets?

- No – in general, making droplets on a chip is harder and slower (fewer droplets / unit time) than other methods.
- Easiest method to make droplets: use a stir plate to stir oil phase (not too fast), add aqueous phase few µL at a time. Make 10^7 droplets in a couple minutes.
  - Broad size distribution, but this is fine for many applications, including emulsion PCR
- Other non-chip approaches to making uniform droplets: vibrating nozzles, coaxial capillaries, etc.
- Use a chip for:
  - Mixing two or more reagents *immediately* before droplet formation
  - Cases (such as HRP evolution example) where assay happens fairly quickly after droplet formation and there may be an advantage to synchronizing droplet formation with detection step.
  - Multi-step processes where you want controlled addition of reagents (numerous literature examples, but may be easier said than done…)
  - Getting pretty pictures for high impact publications???



Uniform droplets jetted at 18 kHz
(Meagher 2011)

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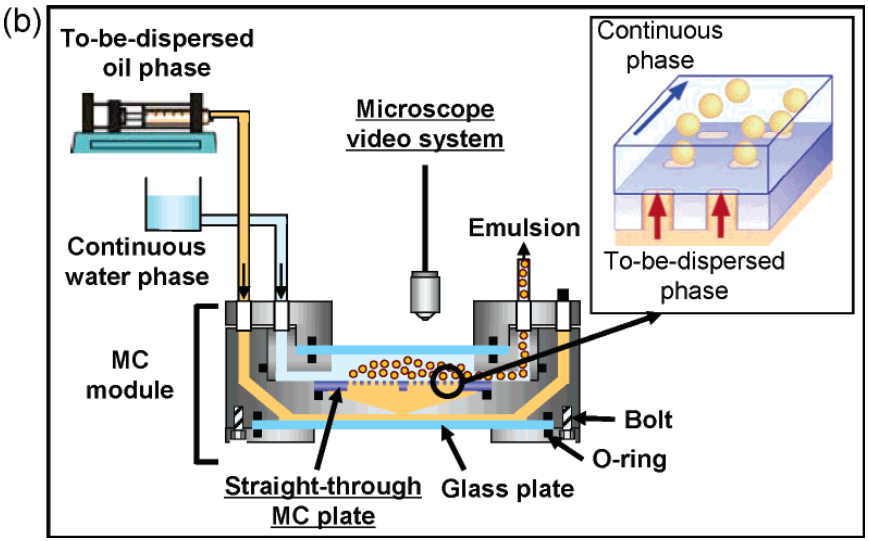
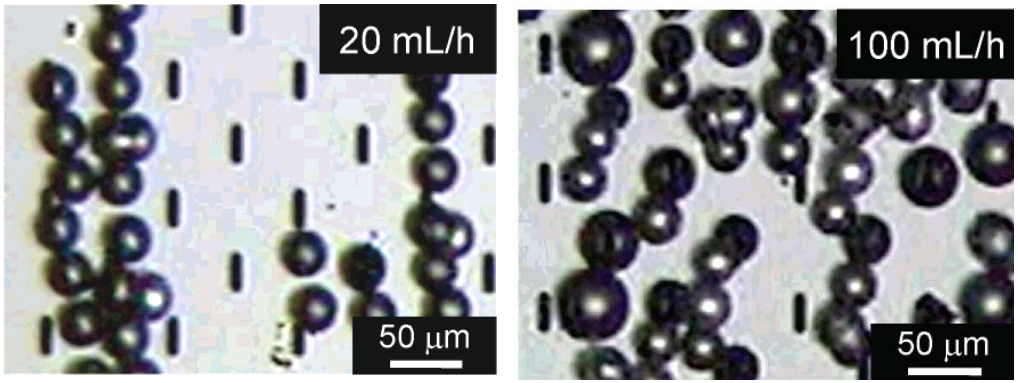
Other microfluidic approachs for making uniform emulsions

Microchannel plate / microporous membrane

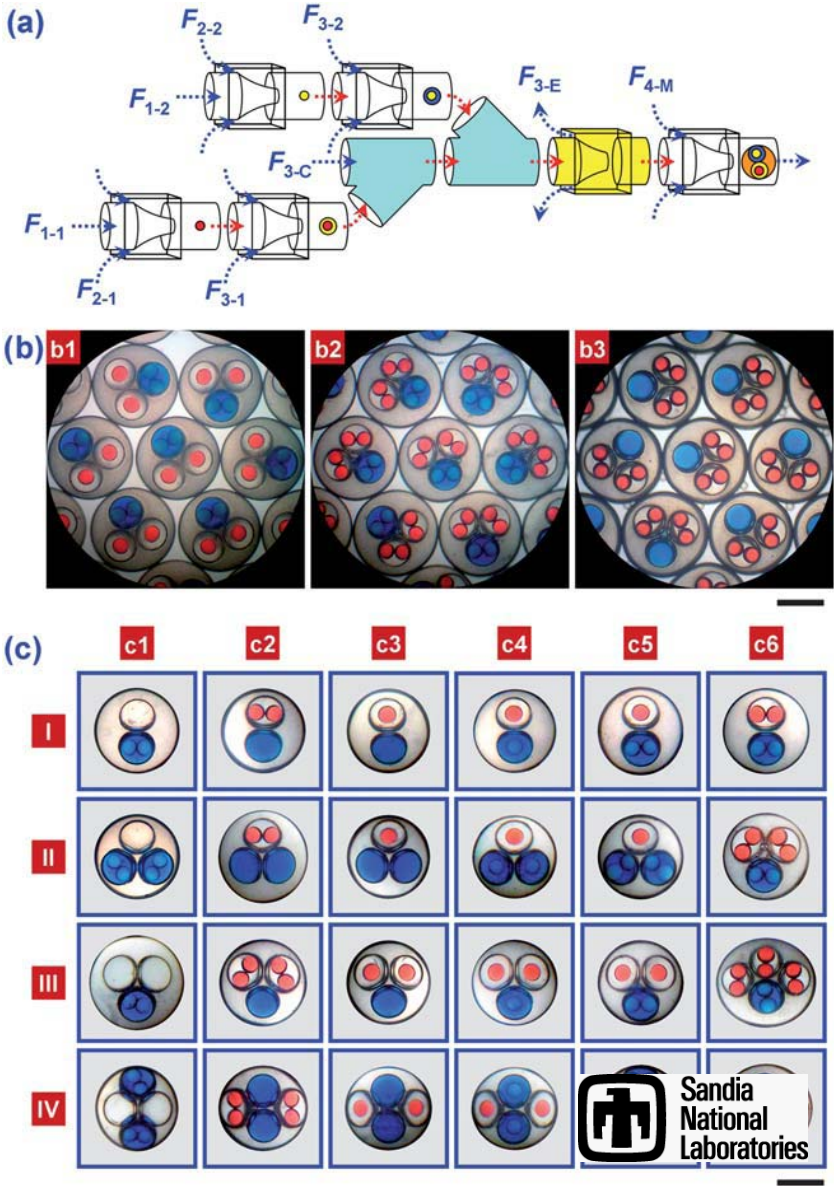




Kobayashi, *Ind Chem Eng Res* 2005

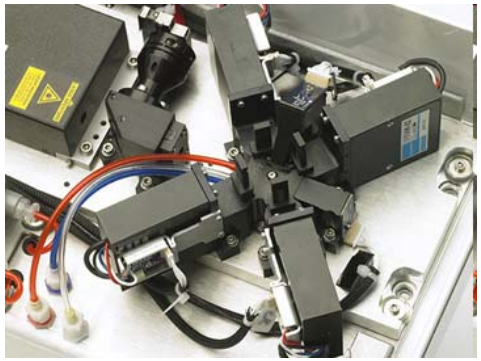
Tapered capillaries and coaxial flows

W. Wang *et al*, *Lab Chip* 2011 (online)



Flow cytometry / FACS on a chip

- Flow cytometry is the best-known “single-cell” analysis – analyze a bunch of cells in single-file line, one cell at a time
  - Usually fluorescence and light scattering properties
- Many examples of chip-based flow cytometry and sorting…
- “Conventional” flow cytometers are inherently “microfluidic”, just not on a chip!
  - Typically use a sheath flow for hydrodynamic focusing
  - Some models use a sheathless micro-flow cell
  - Sorters typically use a piezoelectric nozzle (~50-100 μm) to generate monodisperse droplets (jet breakup mode) which can be selectively deflected into a collection tube



Small benchtop flow cytometer
(analyzer only, no sort)
~30 x 40 x 40 cm, 2 lasers, 6 detectors
~10^4 counts/s
7-log dynamic range
~$30k (?)

www.bd.com

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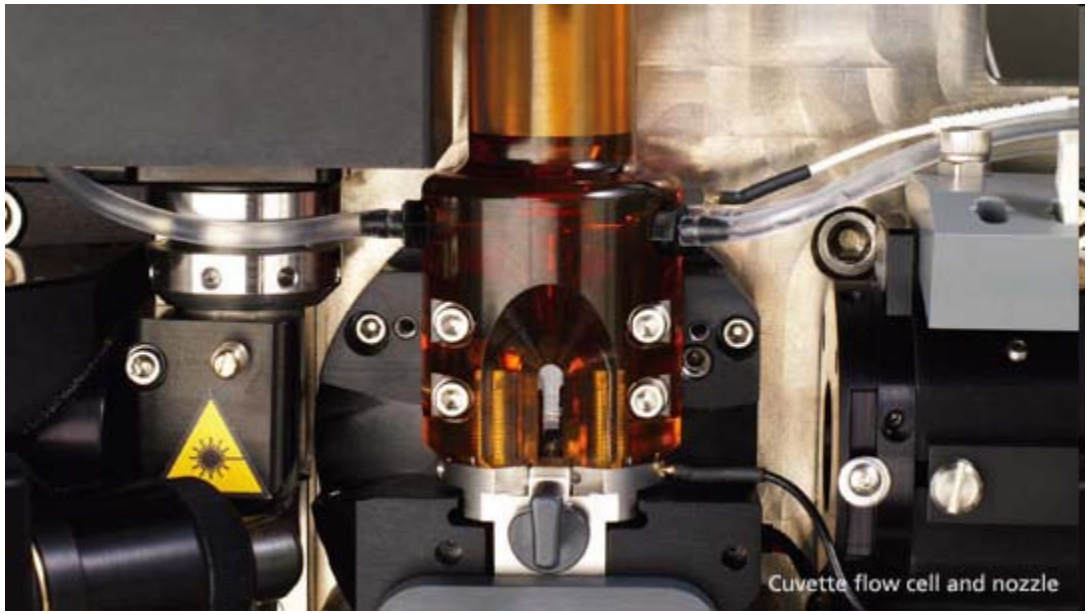
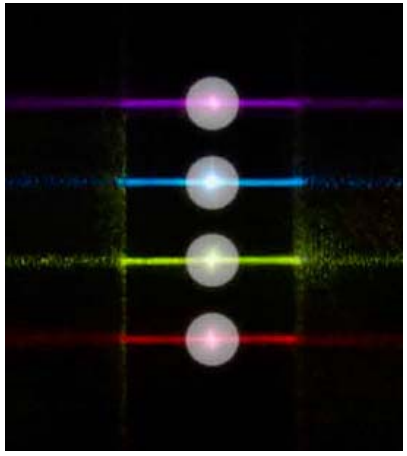


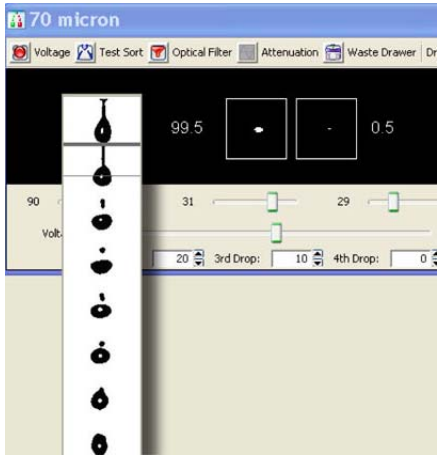
Modern flow sorter

Up to 6 lasers
20 detection channels
~10^5 events/second
Nominal sensitivity to ~100 dye molecules per event
Real-time data reduction and analysis
User defined sort gates Multi-way sorts
Single cell deposition into microtiter plate or user-defined formats
Temperature controlled
Aerosol containment
~ 70 x 70 x 80 cm package



Multicolor analysis

How to pack a lot of detectors into a small space



Droplet generation and sorting

www.bd.com

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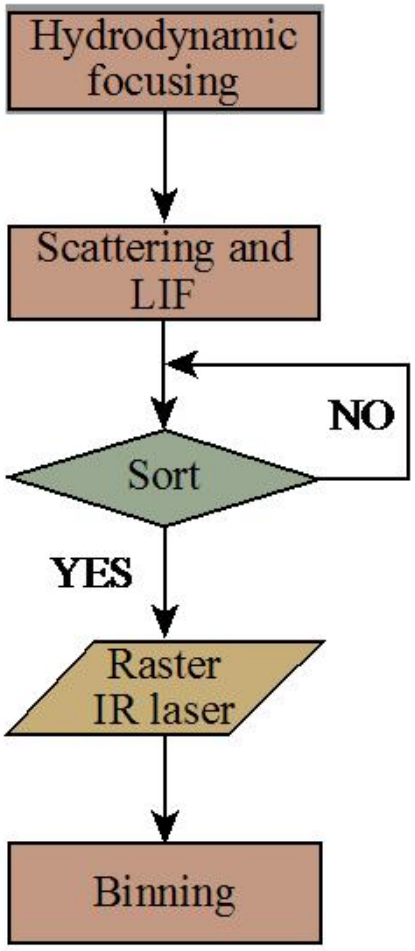
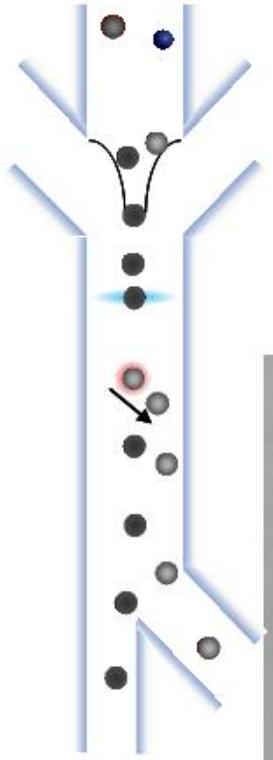
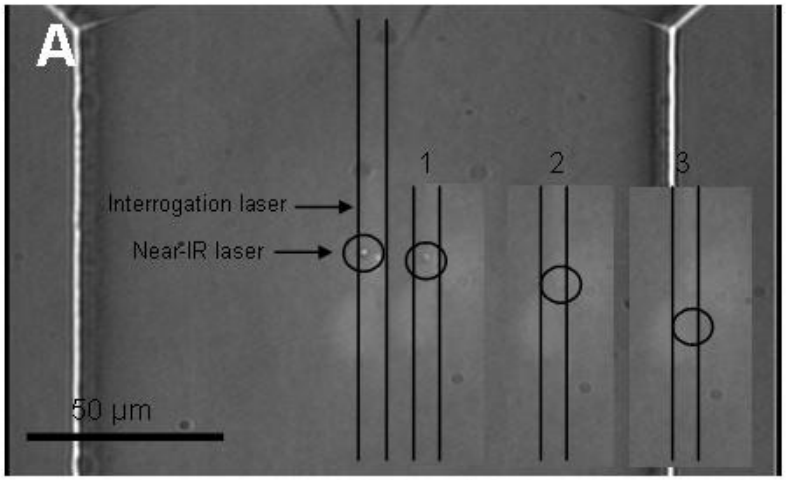
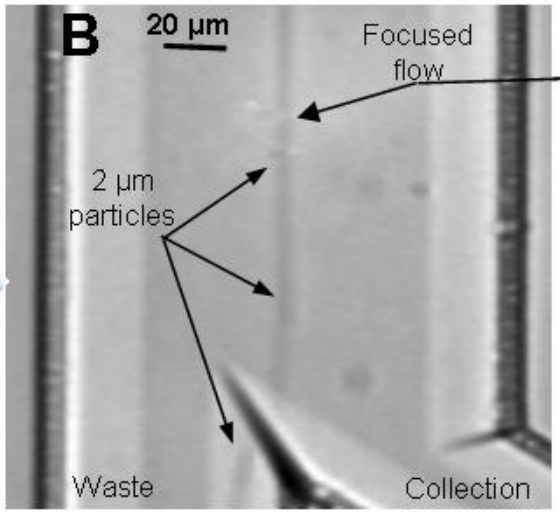
vs… a Chip-based flow sorter

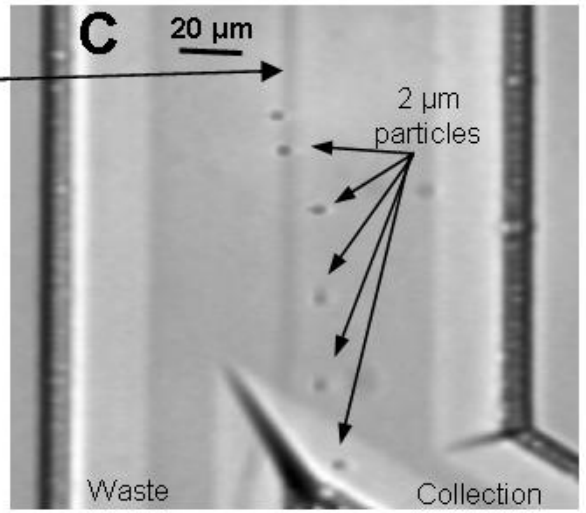
Glass chip, 2D hydrodynamic focusing, 2 lasers, 3 fluorescence channels + FSC
Sort mechanism: Fluorescence-activated actuation and deflection of an optical trap











T. Perroud *et al*, *Anal Chem* 2008

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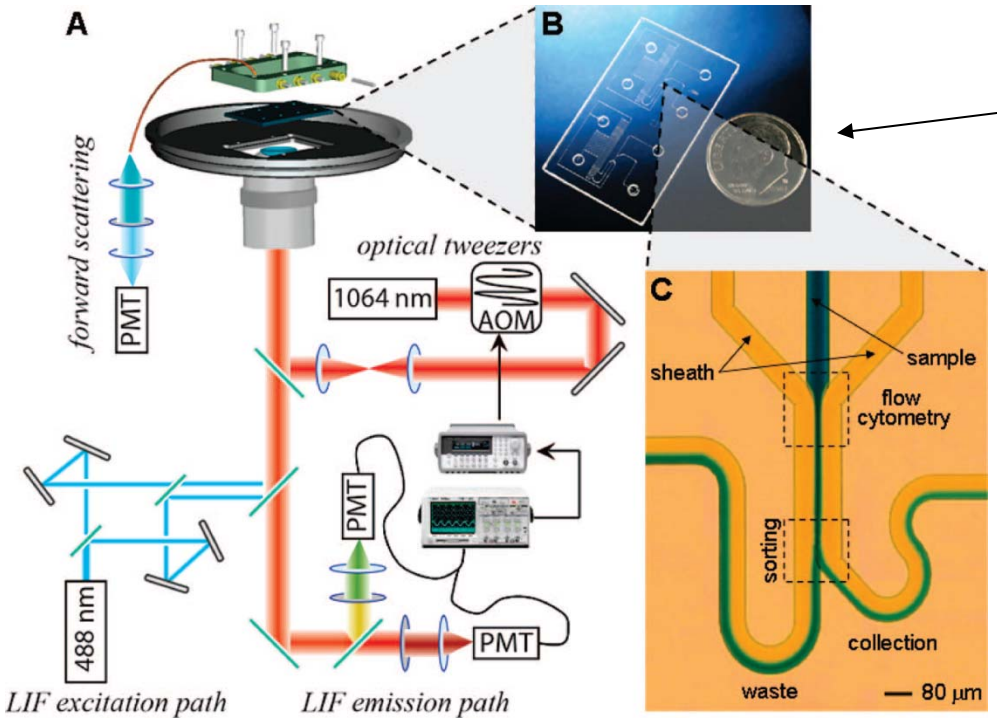
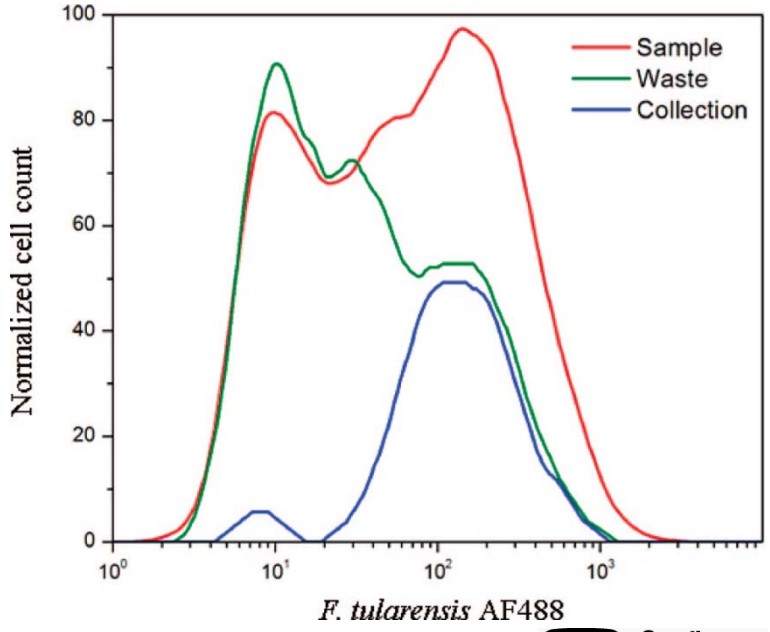


Small chip!
22 x 37 mm

2D hydrodynamic focusing

Macrophages infected with *F. tularensis* (!)
Sorted low-infected from highly-infected cells.
22 cells/s, 93%



T. Perroud *et al*, *Anal Chem* 2008

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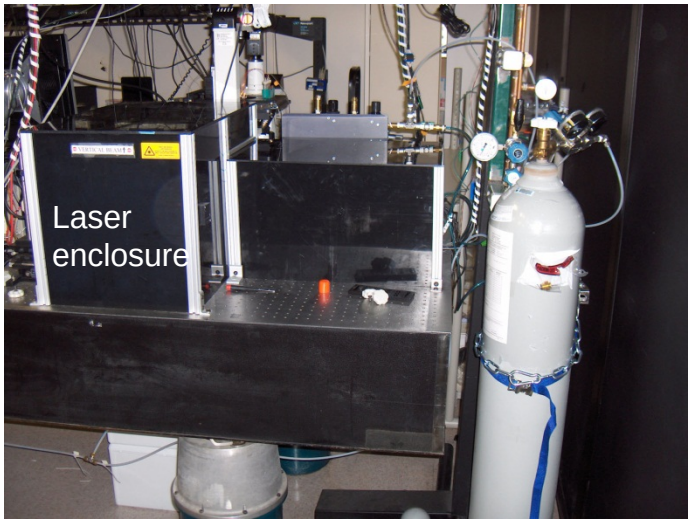
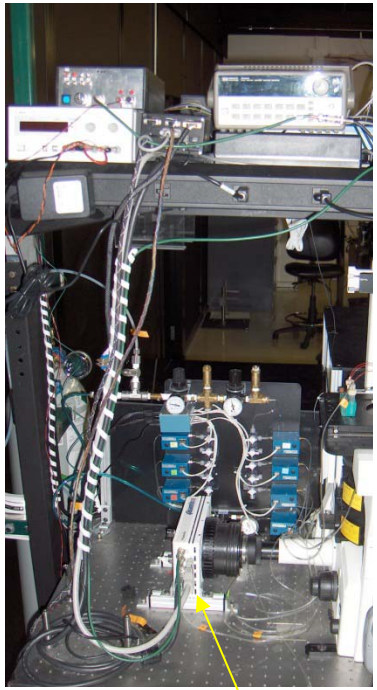
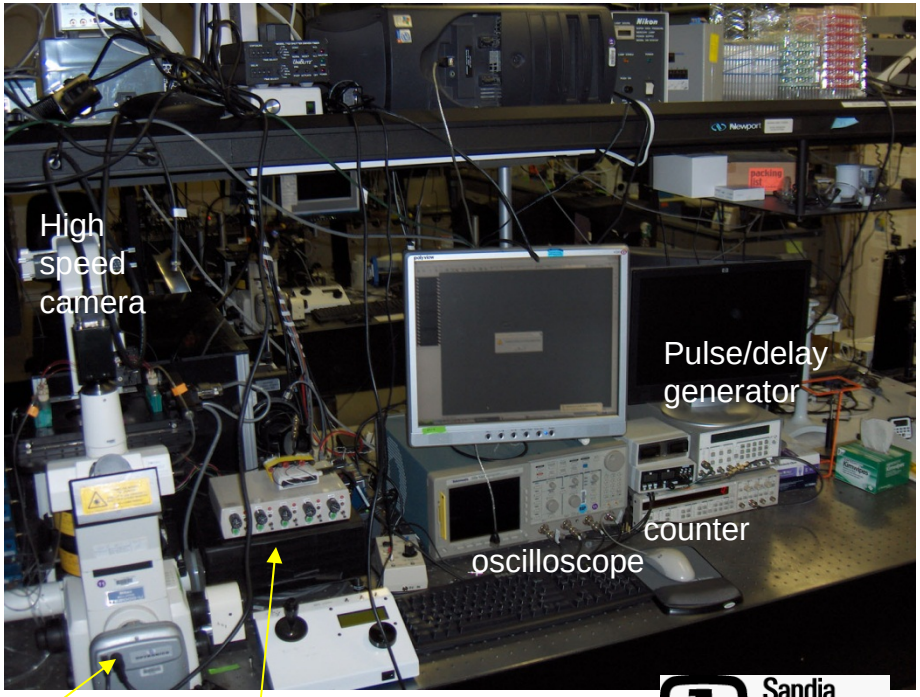
Home-built chip flow cytometer/sorter
2/3 of an optical table!

Cell sorting chip mounted on inverted microscope



Electronics (function generator, power supply)





Pressure sources

CCD cameras

PMT enclosure and power supplies

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Q: Why do I show you this?

A: To illustrate a key point -

- A home-made chip flow cytometer is never going to “beat” a commercial instrument by any “conventional” measure of performance
  - Throughput
  - Sensitivity
  - Dynamic range
  - Number of channels/detectors
  - Sort rate
  - Ease of use
  - Expense (!)

This old (c. 2000) FACScan analyzes 20,000 cells/s with 5 fluorescence and 2 scatter channels

- The commercial instrument will typically beat the home-built instrument *by orders of magnitude*
  - *The commercial instruments represent a multi-billion dollar endeavor spanning several decades, with top-notch expertise in optics, fluidics, electronics, signal processing, programming, and systems engineering, and years of collaborative development with biology and medical researchers!*

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So why bother?

- Conventional flow cytometers are incredibly powerful, and surprisingly flexible instruments for research, *but*…
- Chips provide opportunities for integration
  - Cell culture, prep, labeling, and assay on a single device with good synchronization
- The usual *disadvantages* of a chip (slower, smaller number of cells, lower throughput, *etc*) are an *advantage* in certain circumstances
  - Precious samples with few cells, *e.g.* primary cells as opposed to cultured cell lines; environmental or clinical samples
    - At 10,000 cells/second, you can blow through an entire precious sample just setting PMT gains!
  - Weakly fluorescent samples may benefit from slow transit through a detection spot (“slow” on a BD FACScan is still pretty fast!)
- Chips are potentially a *closed system*, potentially *disposable*
  - A single-use “disposable” flow cell, vs hours of bleaching
  - Sorting without aerosol generation for infectious samples

Other examples of cytometry / sorting on a chip

- Srivastava *et al Anal. Chem*. 2008 : Integration of cell signaling / phosphorylation assay with flow cytometry on a single device (cell culturing, challenge, incubation, fixation and permeabilization, antibody labeling, cell detachment, and flow cytometry).

- Shameless plug: Meagher *et al* talk at MSB tomorrow, on *in situ* hybridization for labeling of uncultured bacteria from environmental samples integrated with enumeration by on-chip “electrokinetic” flow cytometry

- Examples of “active” cell sorting by:
  - Opening/closing integrated valves on PDMS chip
  - Pressure pulse from deflection of piezoelectric membrane
  - Dielectrophoresis
  - Optical trapping

- Not to be confused with “passive” cell sorting
  - Magnetic bead capture
  - Dielectrophoresis
  - Filtration

Photopatterning polymeric materials in microfluidics

- The combination of optically clear devices and complex 2D or 3D layouts makes microfluidic chips amenable to innovative techniques to photopattern functional materials
  - Hydrogels, *e.g.* PEG-diacrylate, polyacrylamide, others
  - Porous polymer monoliths
  - Others?

- Photopatterned materials may serve as structural elements (including fully photopolymerized devices), control elements (*e.g.* pH-responsive hydrogel valves), sieving or chromatographic media, size- or charge-selective barriers, and other functional roles.

- Photopolymerization has also been used to produce novel gel particles, fibers, and other unique structures.

Photopolymerization in chips

- At least two broad categories to consider:
- Phase separation polymerization
  - Highly crosslinked materials
  - porous “monoliths”, often with a “popcorn” or “cauliflower” like morphology, but may also have a “sponge”-like morphology
  - As polymer chains grow, they become incompatible with the casting solvent, and phase-separate, forming dense, solid domains.
  - Heirarchical pore structure characterized by micron-sized voids
  - Commonly used as support for chromatography and immobilized enzyme reactors
- Hydrogels
  - Typically high water content, characterized by thin gel fibers and 1-10 nm sized pores; appear homogeneous at visible length scales (>200 nm)
  - Crosslinked polyacrylamide is a typical example
  - Some use as sieving media for electrophoresis, but this is often better accomplished by replaceable polymer matrices
  - Useful for trapping cells and biomolecules, controlled release, creating structured microenvironments for cells, and creation of nanoporous “filters” for analyte concentration.
- Some overlap between categories, *e.g.* addition of PEG to polyacrylamide can cause “lateral aggregation” or phase separation during polymerization, resulting in a macroporous hydrogel.

Surface attachment of polymer elements

- In glass chips, polymer monoliths and gel plugs should be covalently anchored to chip surface
- This can be accomplished by a silane with a polymerizable group, *e.g.* methacryloxypropyltrimethoxysilane (MAPS).
  - This is also the first step of a traditional Hjertén-style coating
- Perhaps unlike the Hjertén polyacrylamide coating, surface attachment of a polymer *does not* necessarily require a perfectly smooth, uniform monolayer.
- A typical protocol would be to deposit the silane from a ~5% solution in toluene for about an hour, on a reasonably clean and dry chip surface (but not rigorously dry – there needs to be some adsorbed water).
- Another typical protocol would be to use ~5% of the silane in ethanol with a trace of water and acetic acid, for about an hour.
- The protocol that I generally use, which seems strange, but works pretty well is:
  - 2:3:5 (v:v:v) mixture of silane:glacial acetic acid:water (mix the silane and acetic acid well before adding water; vortex for ~30 seconds upon addition of water).
  - Fill channels and flush through, let stand for 30 minutes, making sure to keep the via holes completely wet.
  - Rinse chip surface and channels thoroughly with a solution of 30% acetic acid, followed by water, followed by methanol, and then dry by vacuum.
- Any region where you *don't* photopolymerize a structure can be coated with LPA afterwards, by polymerizing acrylamide w/o crosslinker (APS/TEMED, or photopolymerization) for a Hjertén-style coating.

Methods for photopolymerization

"Contact lithography" methods

- Flood illumination with a simple UV lamp (non collimated) through a photomask
  - Chip placed in contact with mask
  - Resolution of features may be limited to due non-collimated source
  - Can improve a bit by placing target ~12 inches above light source (rays will be mostly on-axis)
- Illumination with a well-collimated UV source, *e.g.* mask aligner
  - Better resolution due to collimated source
  - May be easier to track intensity with this type of source

Theoretical resolution limit b_{min} given by $2b_{\min} = 3\sqrt{\lambda(s + z/2)}$

λ = wavelength, s = spacing between mask and channel, z = depth of channel
Actual resolution may be lower due to poor collimation, diffusion of radicals outside of illuminated zone, scatter of UV light from monolith particles, heating & stray polymerization due to intense irradiation, irregularities on chip/channel surface, etc.

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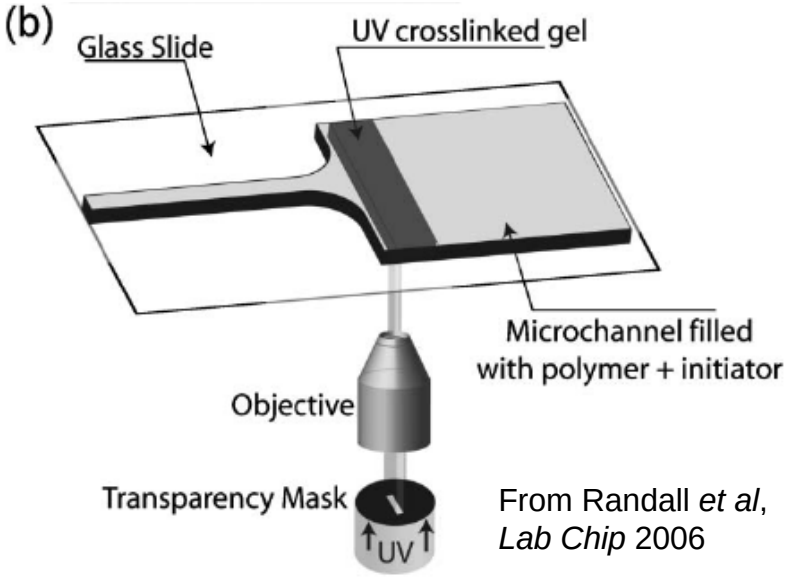
Methods for photopolymerization

“Projection lithography” methods

- Projection through a photomask on microscope
  - Photomask placed in path of collimated mercury arc lamp or UV LED
  - Chrome mask is preferred due to intense heat if using a traditional mercury source, although this may be mitigated if using an LED source or metal halide source with liquid light guide coupling
  - Image of photomask is projected onto target through objective (with reduction in feature size)



From Randall *et al*, *Lab Chip* 2006

- Laser projection
  - UV laser (*e.g.* 355 nm DPSS) is projected through a slit, to cut off gaussian “edges”
  - For “elongated” features – beam is focused through a cylindrical lens to create a laser sheet
  - Beam shape can be visualized and optimized using a chip filled with a UV-excitable, visible-wavelength fluorophore, *e.g.* Rhodamine B, and a CCD video camera.
  - Longer features can also be realized by stepwise polymerization, or by scanning the beam (motorized stage or scanning mirrors), although excess production of free radicals may reduce resolution.

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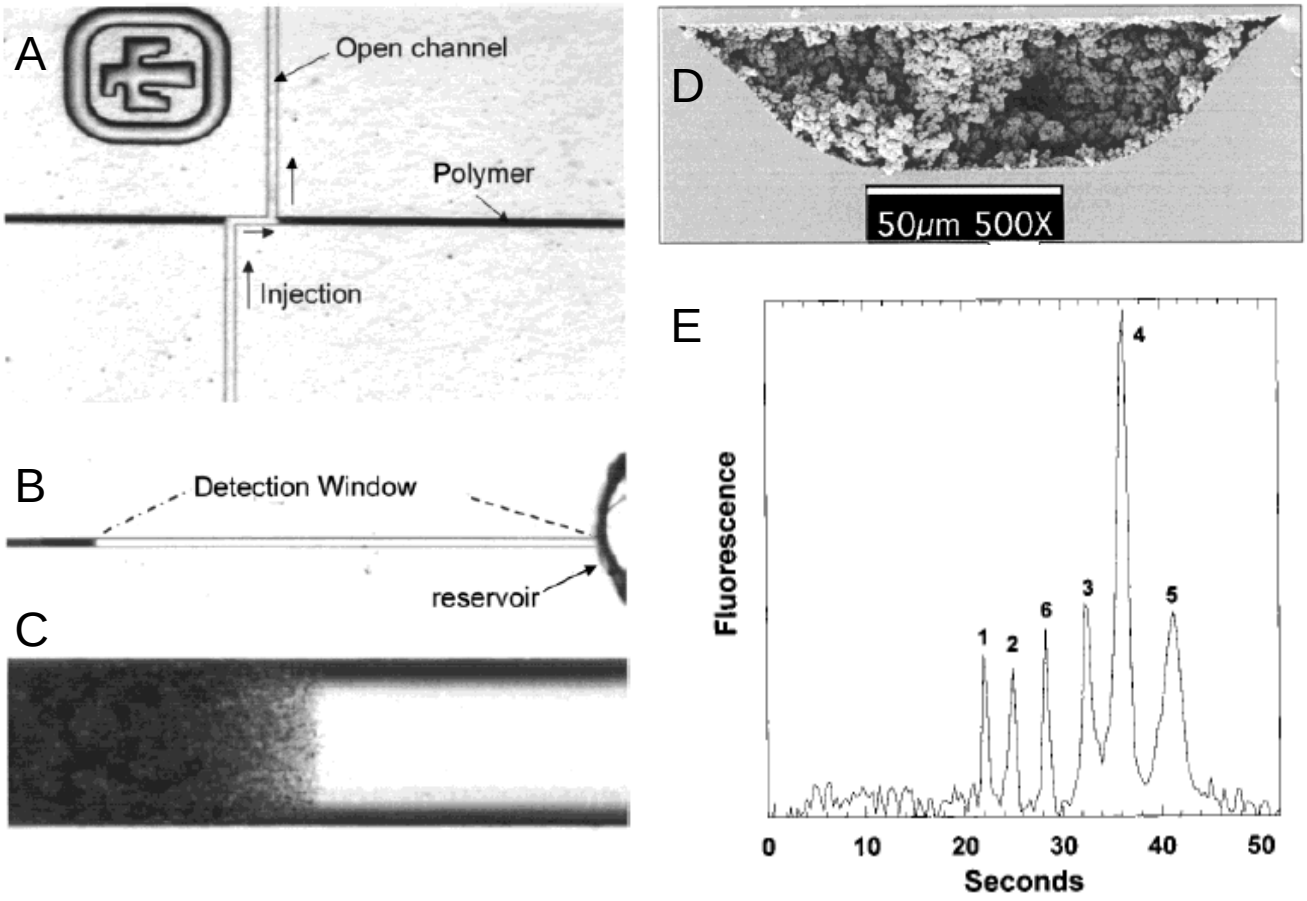
Porous polymer monoliths by phase-separation polymerization



Acrylate polymer monloith patterned using a mask and a UV lamp (flood illumination). Polymerization mixture is 1:2 monomer mixture:solvent mixture. Solvent mixture is 1:3:1 ethanol:acetonitrile:phosphate buffer. Solvent composition affects polymer MW or domain size at onset of phase separation. (A,B,C) Patterning of monolith to keep injection arms and detection window void of polymer in an offset double-T chip (D) SEM micrograph of channel filled with porous polymer monolith, shows 1 µm average pore size. (E) Reversed-phase electrochromatography of NDA-labeled peptide standards in a microchip with negatively charged lauryl acrylate monolith. Figures from Throckmorton *et al*, *Anal. Chem.* 2002

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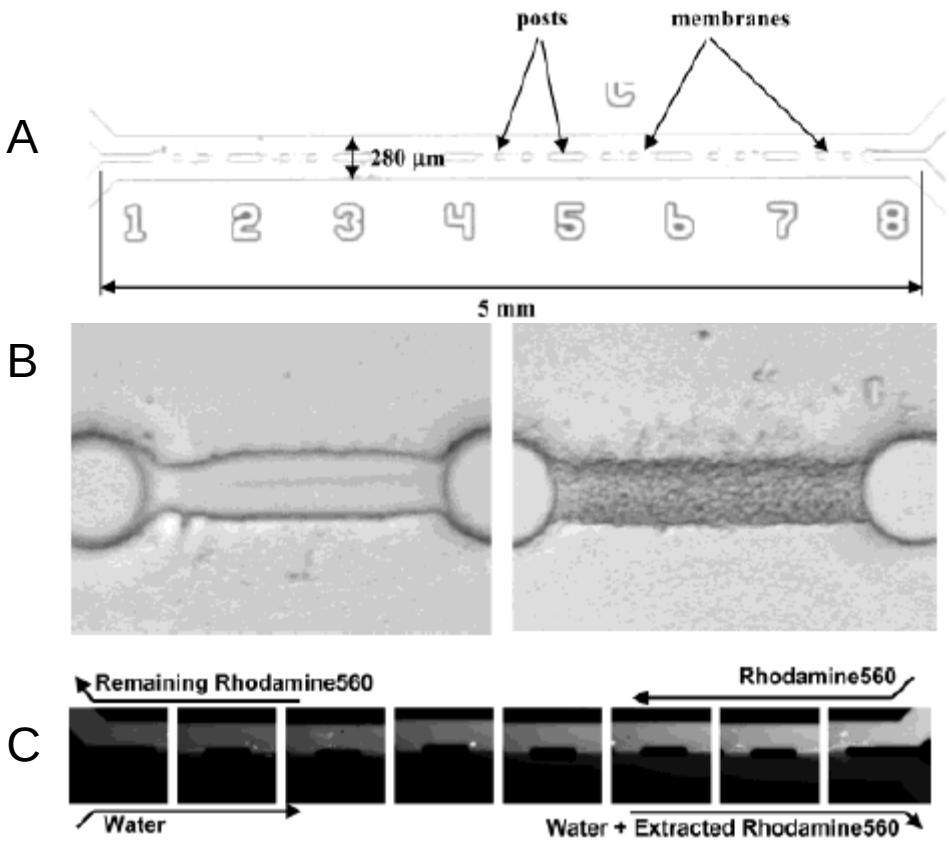
Laser-patterned membranes



Porous polymer monolith dialysis membrane formed by laser patterning of zwitterionic acrylate monomers. (A) Channel layout, showing position of membrane down the center of a long microchannel, with microfabricated "posts" for support. The membrane was patterned between posts in segments of ~600 µm at a time. (B) Optical micrographs of membrane segments between posts. The image on the left is of a low MWCO membrane (<5700 Da), while the image on the right shows a higher MWCO membrane. Pore sizes were controlled by varying the compatibility of the solvent used in polymerization. (C) Fluorescence micrograph showing removal of Rhodamine 560 from a flowing stream by countercurrent dialysis across the microfabricated membrane.

Figures from Song *et al, Anal. Chem.* 2004

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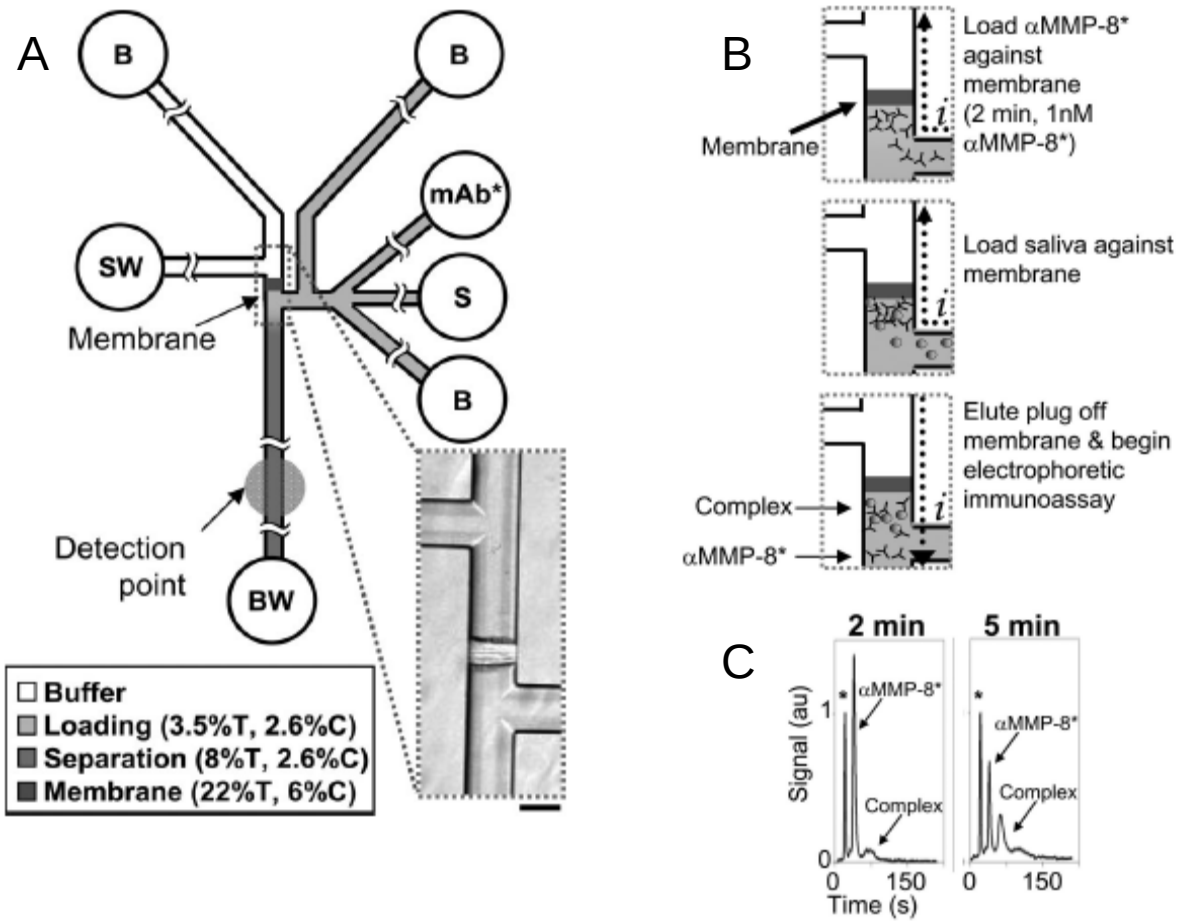
Laser-patterned polyacrylamide hydrogel membrane for electrophoretic preconcentration of proteins integrated loading gel and separation gel patterned by UV exposure through mask



Schematic of chip for electrophoretic immunoassay with integrated preconcentration membrane. The different zones (Loading, separation, membrane, and buffer) are formed by successively filling the chip with different monomer compositions and photopolymerizing specific regions. Wells designations are: B = Buffer, S = Sample, mAb* = Fluorescently labeled monoclonal antibody, SW = Sample Waste, and BW = Buffer waste. (B) Schematic of electrophoretic preconcentration and mixing of reagents at integrated membrane. Current flow is indicated by arrow labeled *i*. (C) Electropherograms showing formation of substantial immune complex with 5 minutes of preconcentration time. The peak labeled (*) is a protein internal standard.

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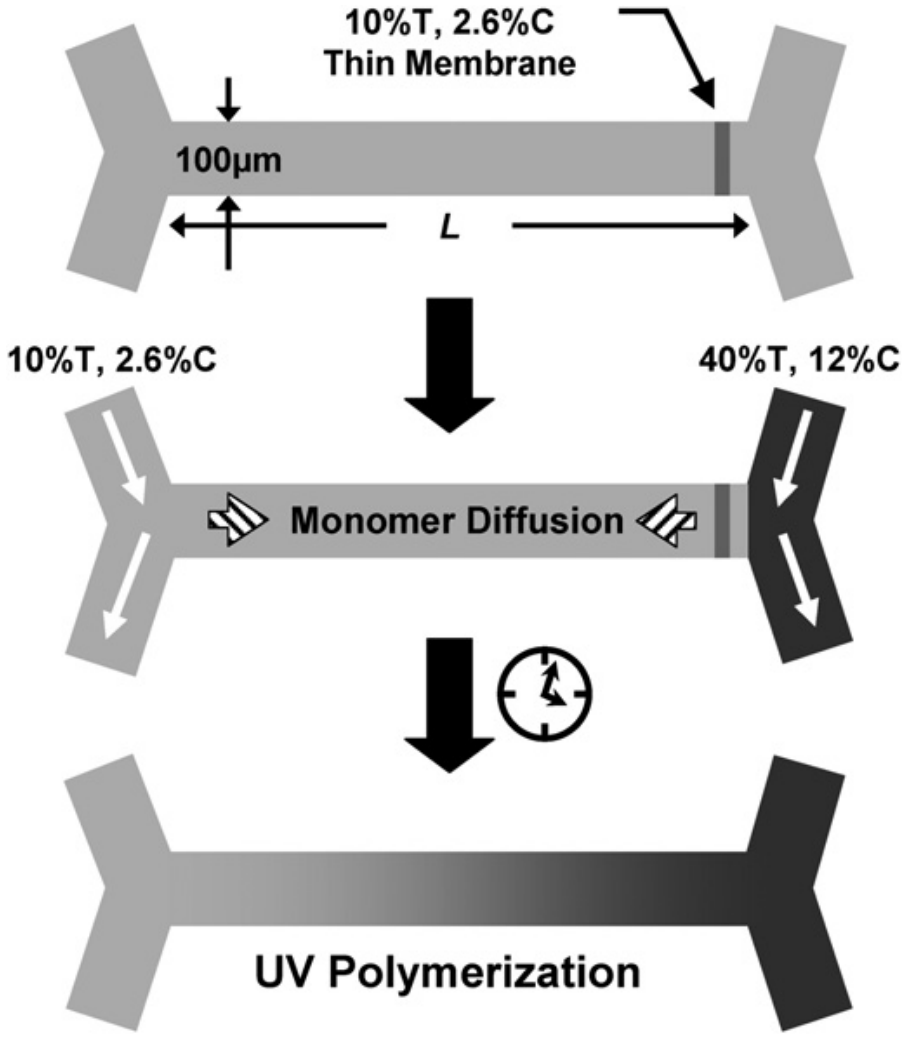
Formation of an immobilized gradient by diffusion and photopolymerization



Channel is filled with 10%T, 2.6%C monomer solution with photoinitatior (2 mg/mL VA-086), and a thin plug is polymerized at one end of the channel, effectively blocking hydrodynamic flow in the channel while permitting diffusion of small molecules.

The two arms of the device are gently flushed with solutions containing two different concentrations of acrylamide/bis acrylamide and photoinitiator (gravity-driven flow, overnight). Monomers diffuse freely across the channel, developing a steady-state linear concentration profile, with no hydrodymaic flow in the channel.

Flood illumination is used to photopolymerize the monomer solution, creating a fixed gradient of acrylamide concentration (pore size gradient).

From Sommer *et al*, *Lab Chip* 2009

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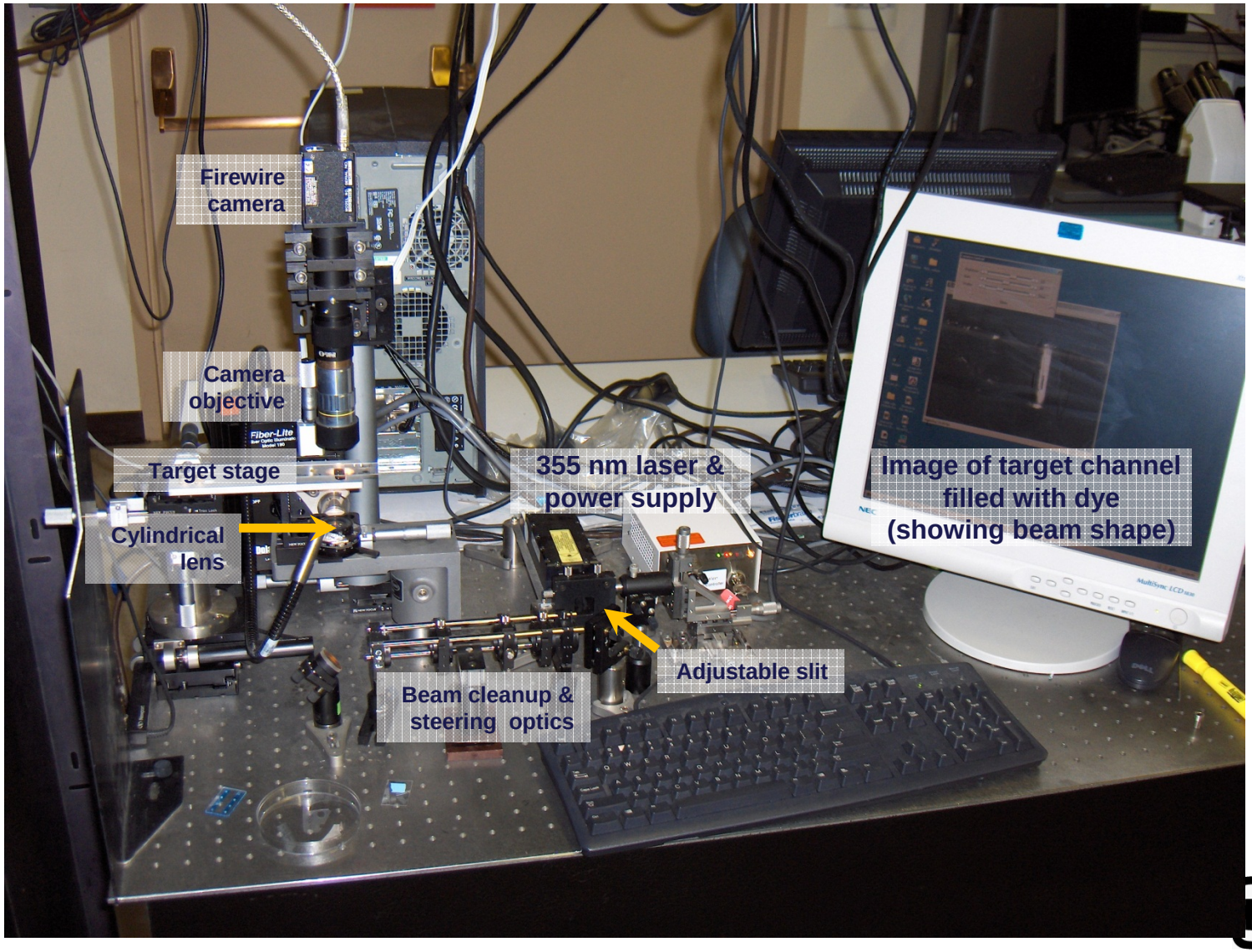
Laser projection photopatterning



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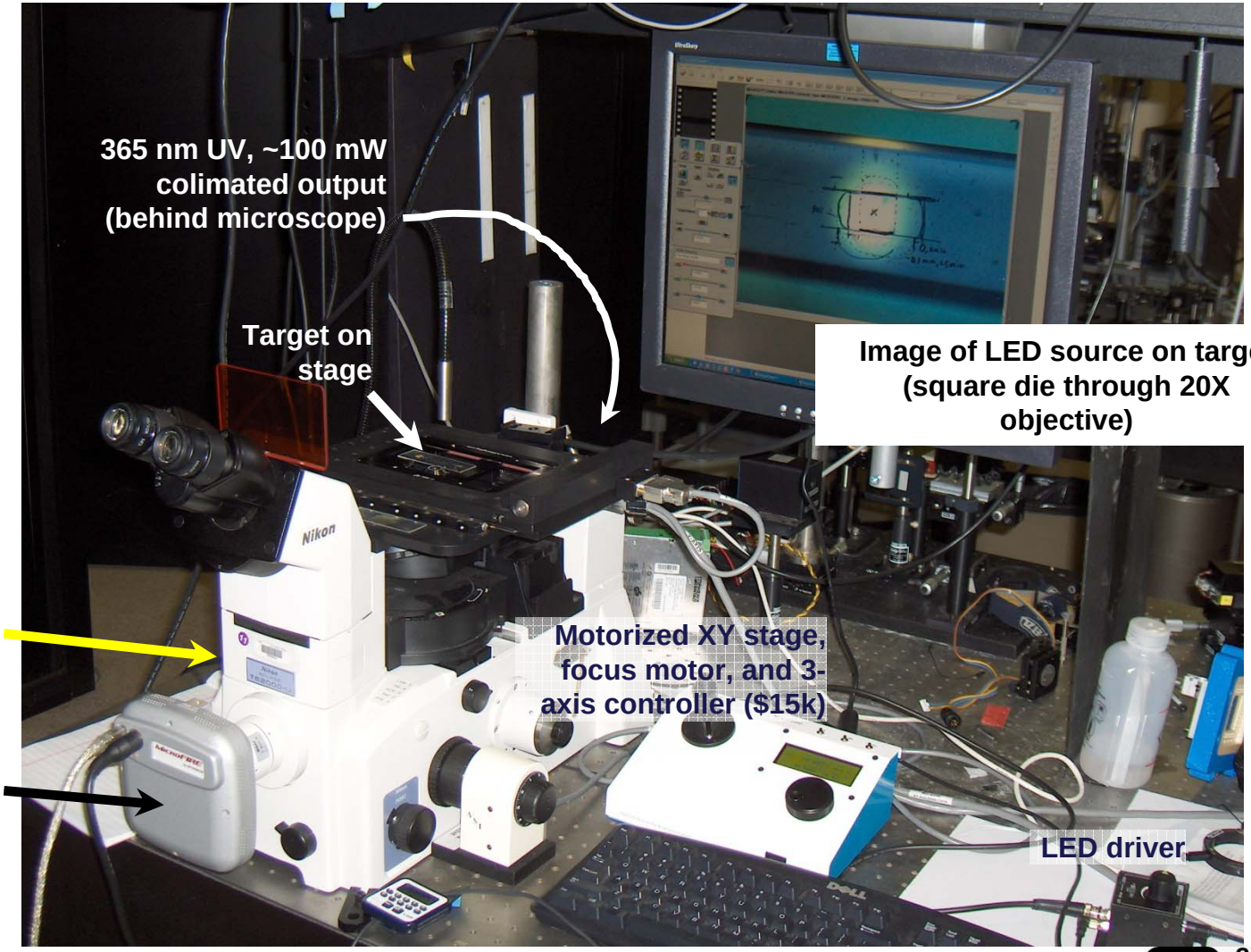
Microscope-based photopatterning (UV LED)



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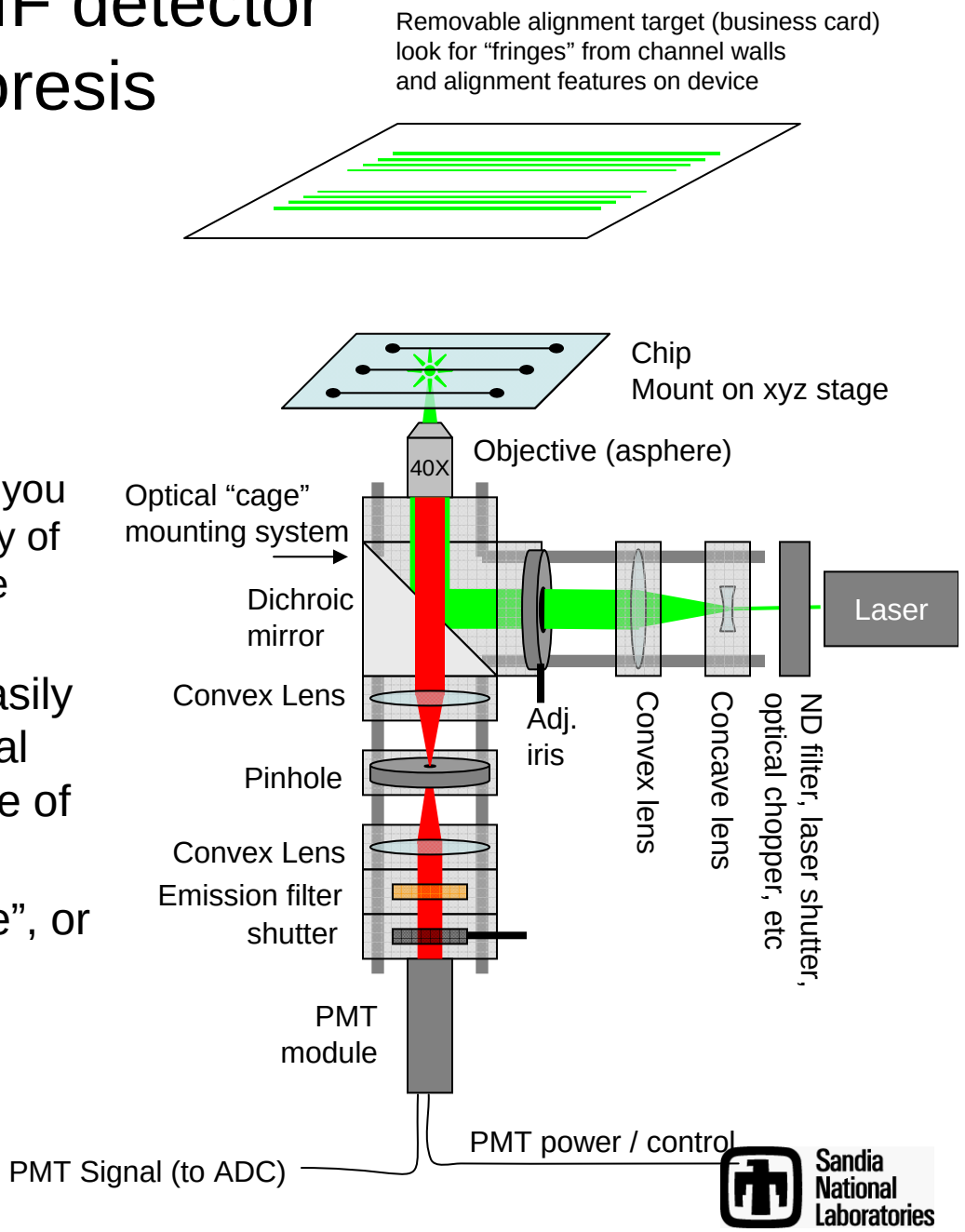
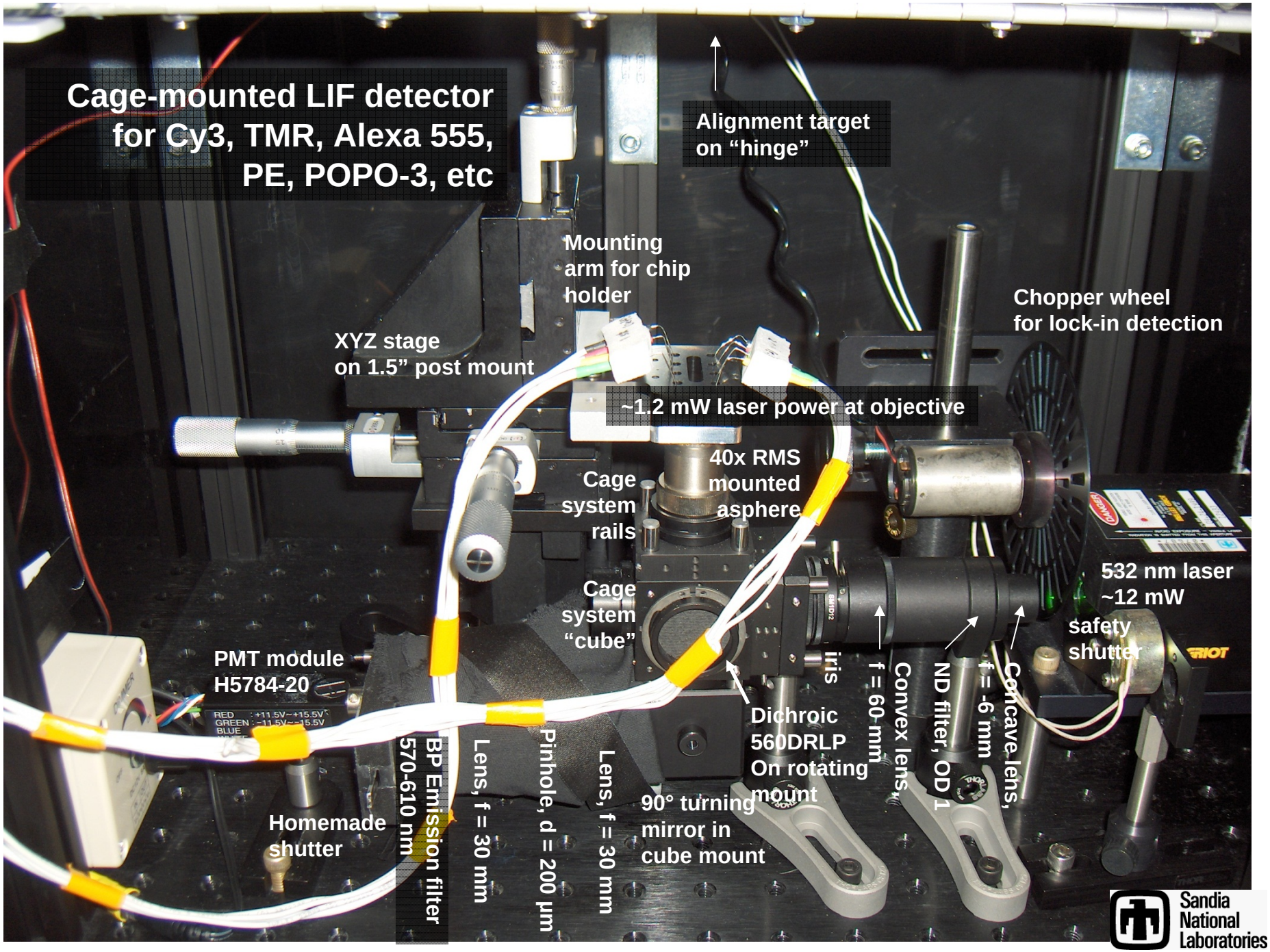
Tips for photopolymerization

- For high resolution features, at least two factors are important:
  - Optical setup / diffraction limits
  - Chemical limitations: diffusion and reaction of radicals outside of illuminated zone.
- With respect to “stray” polymerization, a couple of observations:
  - Typical things you might do to prepare for a polymerization, such as sparge or sonicate under vacuum to remove oxygen, or passage through a column to remove inhibitors, is not necessarily helpful here.
  - You may actually want a little bit of residual oxygen or chemical inhibitors to prevent polymerization outside of the illuminated zone.
  - For laser polymerizations – I typically add 150 ppm of hydroquinone (a radical scavenger). It slows the polymerization a bit, but seems to improve feature shape.
  - Need to optimize photoinitiator and radical scavenger together.
- Also need to have no hydrodynamic flow, to allow polymer to gel
  - I personally have good enough results by just carefully pipetting to fill all via holes equally.
  - Others have tried placing a coverslip over the chip, or tape over via holes, or filling wells with a viscous polymer solution after filling the chip… whatever works for you.

How to build a simple LIF detector for chip electrophoresis

- For simplicity and convenience of alignment, people often build LIF detectors around microscopes
  - This is unnecessary, and ties up an expensive, versatile research tool.
  - This is potentially hazardous unless you are careful to eliminate the possibility of even low-level laser light through the eyepieces focused on your retina
- A fully functional LIF detector can easily be constructed out of common optical mounts and components for a couple of thousand dollars.
- Commercially available “cage”, “tube”, or “rail” – style optical mounts simplify construction and alignment of components.




Cage-mounted LIF detector for Cy3, TMR, Alexa 555, PE, POPO-3, etc
Alignment target on "hinge"
Mounting arm for chip holder
Chopper wheel for lock-in detection
XYZ stage on 1.5" post mount
~1.2 mW laser power at objective
40x RMS mounted asphere
Cage system rails
Cage system "cube"
532 nm laser ~12 mW
safety shutter
PMT module H5784-20
iris
Convex lens, f = 60 mm
ND filter, OD 1
Concave lens, f = -6 mm
Dichroic 560DRLP On rotating mount
90° turning mirror in cube mount
Homemade shutter
BP Emission filter 570-610 nm
Lens, f = 30 mm
Pinhole, d = 200 µm
Lens, f = 30 mm
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Choice of Laser

- Factors in the decision:
  - Do you have a spare laser or filter sets laying around?
  - Is there a particular fluorophore that you are committed to using?
  - UV and Infrared wavelengths present additional (but surmountable) challenges in alignment and safety.
- Laser wavelengths
  - Inexpensive laser diodes (typically red and NIR, e.g. 635-700 nm)
  - Slightly more expensive laser diodes in violet and short blue (*e.g.* 405 nm, 445 nm)
  - Less expensive HeNe gas lasers – 543 nm, 594 nm, 633 nm, wavelength-selectable (turnkey packages, no external cooling required)
  - More expensive gas lasers (Ar-ion 488 nm or 514 nm, Kr-Ar 488/568/647 nm), usually large and require cooling fans
  - Diode-pumped solid state lasers
    - usually less expensive than gas lasers but more than laser diodes
    - Visible wavelengths are often frequency-doubled or –tripled versions of IR lasers: 355 nm, 488 nm, 532 nm, 561 nm, etc.
- Power output
  - Usually <10 mW is fine for chip electrophoresis
  - More power leads to more signal, but faster photobleaching, possibly more “background” in fluorescence measurements from Raman scattering
  - In US: 5 mW is the dividing line between Class IIIa and IIIb, entails different levels of engineered controls for safety.
- Pre-coupled to an optical fiber is always convenient

Filters and Optics

- Neutral density filters may be useful for attenuating beam for safety during alignment, or generally to decrease laser power into a useful range.
  - Place at slight angle so beam doesn't reflect back onto laser aperature
- Dichroic: choose one that is a close match to your laser. Vendors include (but not limited to) Chroma, Semrock, Omega Optical.
- Emission filter: choose newer-generation filters with high transmission in pass band and steep, well-defined cutoff wavelengths.
- Optional: laser line filter to cut out any stray reflected laser light that makes it past the emission filter
- Objective lens – a simple mounted aspheric lens (~40X, 0.55 NA) is fine, inexpensive choice (<$100)
  - An imaging-quality microscope objective is overkill.
  - Make sure the objective lens has appropriate AR coating for your wavelength.
  - If you are using a microscope objective – determine whether it is an image-forming objective (common on older microscopes), or infinity objective (most modern objectives from Nikon, Olympus, Zeiss, Leica, *etc*.)
- Expand beam to fill back aperature of objective
  - For a nice beam shape, a Galilean beam expander (concave / convex lens pair) is fine
  - If beam needs some cleanup, a Keplerian telescope (convex lens / pinhole / convex lens) can help, but this takes more real estate.
  - For fiber-coupled lasers – beam-expanding fiber couplers are available.

Confocal pinhole?

- A well-aligned spatial filter or pinhole on the emission side cuts out light originating from outside the focal plane of the chip.
- The aperature of an optical fiber can serve as a pinhole.
- The pinhole size should be chosen based on the wavelength of light, the magnification of the objective, and the degree of “confocality” you are trying to achieve.
- “Optimal” size = size of diffraction limited spot × magnification of objective × any any additional magnification elements: $d_{pinhole}$ $0.61\lambda/NA \times M$
  - e.g. for M = 40X, NA = 0.55, λ = 550 nm, d ~ 25 µm
- Any smaller than the “optimal” simply cuts out signal without improving depth resolution
- The pinhole must be aligned in X, Y, and Z dimensions at the rear focal plane of the objective (or at focal plane of “tube lens” for an infinity objective)
- A larger pinhole (*e.g.* 100 µm) will be more forgiving to align, but will let more light in.
- General consideration: a pinhole is not so hard to align with visible wavelength emission; it becomes a bit trickier (but doable) with NIR emission (Cy5, Alexa 647, etc).
- Including a confocal pinhole will somewhat increase the total size of your optical setup (may be inconvenient in a miniaturized/portable device).

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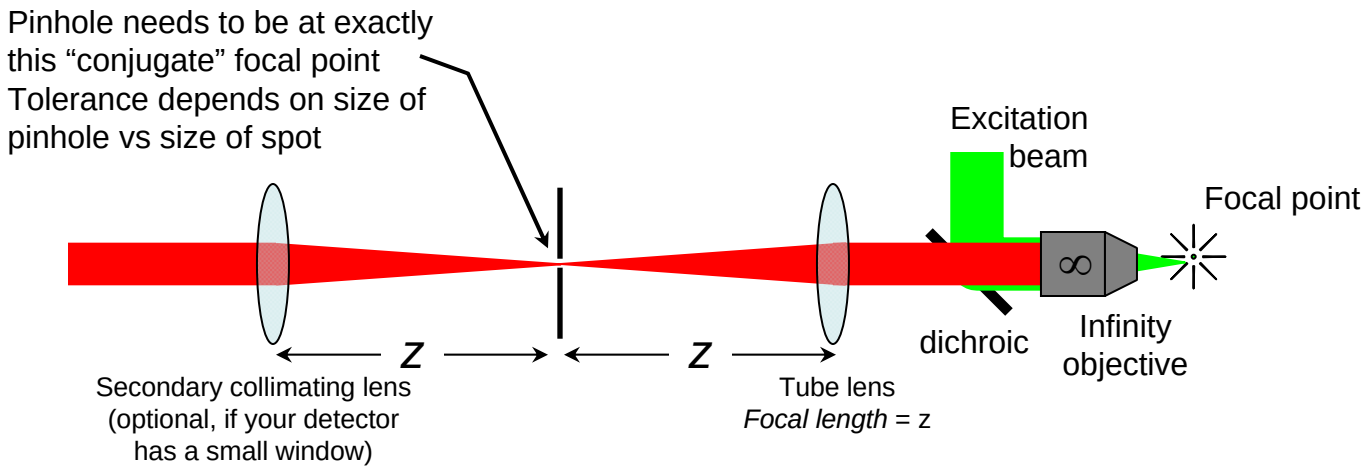

To align a pinhole at a *visible-wavelength:*

1. Pinhole should be mounted in a translating mount with XY translation at least, preferably XYZ translation
2. Fill a dummy chip with channel of same height as your real chip with something very bright - a suspension of brightly dyed microparticles (0.2 µm or smaller, well below diffraction limit of objective lens) may be better than a dye solution
3. Align chip so that laser is focused on channel (use your alignment target to looking at fringe patterns)
4. Start with the pinhole mounted far away from the objective (and "tube lens" if using an infinity objective).
5. Hold a small piece of paper with tweezers beyond the tube lens, and slowly move it backwards. You should see the fluorescence emission on the paper, converging to a point a certain distance from the tube lens (should be close to the focal length of the tube lens)
6. Try to position the pinhole at approximately this Z position.
7. Hold your piece of paper behind the pinhole, and adjust the X and Y position until the emission is brightest.
8. Move the paper back slowly from the pinhole. You should see the spot expanding as you move the paper back.
    a. If the beam appears "collimated" (*i.e.* doesn't expand) as you move the paper back – the pinhole is at the wrong "z" position relative to the tube lens; you are letting only the on-axis rays through.
9. Once you think you have the right position – try adjusting the focus on your device. As you move out of the plane of the channel, your fluorescence spot should go away
    a. If you continue to see something like a bright point with a ring around it, return the focus to within the channel, and adjust the pinhole.

To align a pinhole at an *invisible* wavelength:

I've never done it. An IR indicator or IR viewer may help (?) if you use a "bright" enough test sample. An IR-sensitive video camera placed in the image plane may also help. Otherwise you may just need to optimize signal on a photodetector while you fiddle with the pinhole. Good luck…

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Detectors

- For simple, single-color LIF, a PMT module is a good choice.
  - High gain, fast response, fairly reliable, but can be damaged by overexposure when powered up.
  - Modules include high voltage power supply and voltage divider, optionally a current-to-voltage amplifier, in a compact enclosure.
  - The end-user generally provides a low-voltage power supply with resistance or voltage programming to control PMT gain.
- A variety of configurations of PMT are available
  - Form factor (head-on or side-on, overall size and shape), output (current or voltage), photocathode material, etc.
- A good all-purpose compact PMT module for visible-to-NIR LIF detection was H5784-20 from Hamamatsu, but has been discontinued.
- Most PMT modules don't come with integral shutters
  - A good practice to include a shutter in your setup
  - Keep power supply off when not in use.
- Other detection options: PD, APD, CCD or multi-anode PMT for spectral detection, APD or PMT with photon counting, etc.
  - More on this later.

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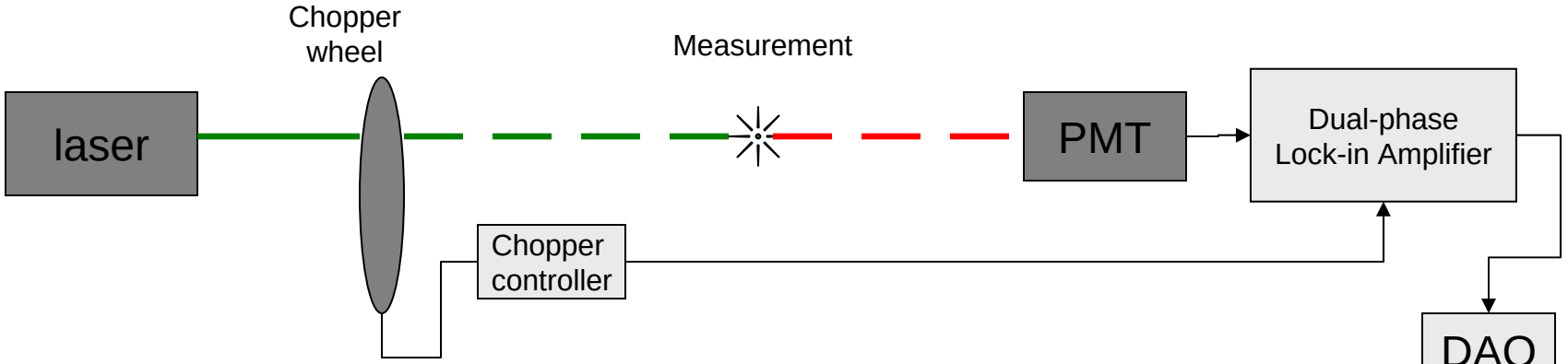
Enhancing signal-to-noise by lock-in detection

- As long as it is powered up, the PMT always has a certain amount of “thermal” noise or dark current, irrespective of whether it is seeing any light.
- When the PMT shutter is open – any ambient light (even if the lights are off) can also contribute to the signal.
- A **lock-in amplifier** is a general-purpose tool for finding a small signal at a known frequency buried in broad-spectrum noise
- For fluorescence detection, we can use this to our advantage by modulating the excitation at a known frequency, and then isolating only the portion of detector signal that occurs at that same frequency.
  - Simplest way to do this – an optical chopper with a frequency-controlled motor. The motor sends a continuous “reference” signal to the lock-in so that the lock-in always “knows” what frequency to look at.
  - A “dual phase” lock-in is most useful as it automatically “knows” the phase lag of the signal.
- The lock-in amplifier is good at rejecting “continuous” sources of noise
  - You can turn room lights on and off, and not even notice…
  - The PMT is still “seeing” all the photons from the room light (at least the ones that make it through the filters), so this is not ideal, but short-term it won’t hurt your experiment.



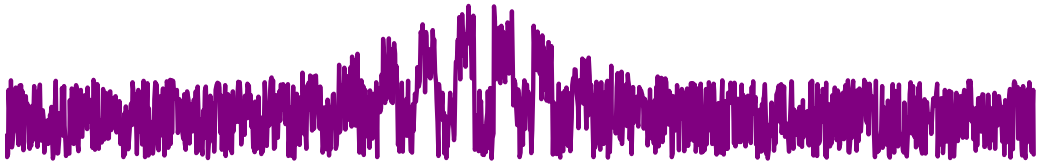
Modulated Laser (also used as reference for lock-in, but phase shifted)

Modulated fluorescence signal

+

PMT "noise"

Overall PMT output (Lock-in signal input)



Lock-in output (reconstructed peak)

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Lock-in amplification: Yeah or Nay

- Lock-in amplification can help with uncontrolled sources of noise when your “real” signal (“fluorescence” photons) are not scarce
  - Optical modulation cuts down your excitation light by ~50%, can compensate by increasing laser power if necessary
- Optical modulation decreases the range of detection frequency
  - *i.e.* if you chop light at 100 Hz, don’t expect to acquire data at 200 Hz!
- Lock-in amplification *does not* help with “background” signals that are correlated to the excitation light
  - Stray scattered excitation light that makes it to your detector
  - Raman scattering from water
  - Autofluorescence of sample or device
- A traditional lock-in amplifier is a bulky, moderately pricey piece of equipment
  - “Miniaturized” or solid-state lock-in modules are available, but may have decreased frequency bandwidth or signal range
  - Fluorescence measurements are typically modulated at <1000 Hz, so frequency bandwidth is usually not limiting
  - PMT output voltages (signal + noise) can be “high”, this may limit utility of miniaturized lock-ins
- An optical chopper is a “moving part” which can be clumsy for portable setups
  - TTL or other modulation strategies are possible for some CW lasers, esp diode lasers
- Lock-in detection (with full-sized instrument) becomes impractical for multiple channels (need 1 LIA per channel)

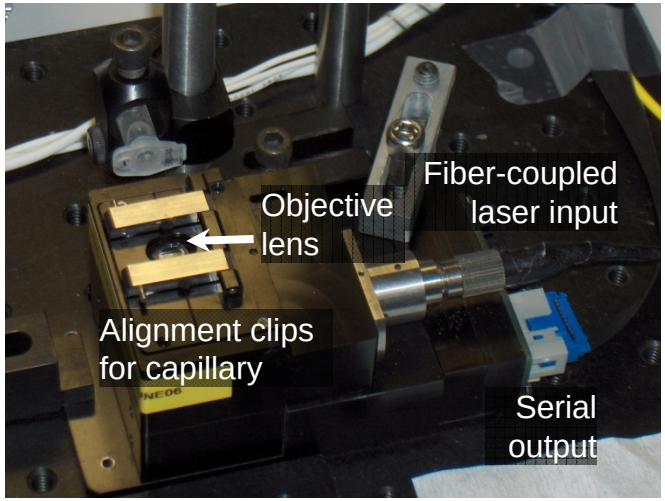
Multi-channel detection options

- Multiple dichroics and bandpass filters to split fluorescence among multiple detectors
  - Easy enough for 2 channels, becomes clumsy for >4 channels
  - Employed in some commercial instruments such as MegaBACE DNA sequencer, and some academic groups including Mathies 4-color DNA sequencing.
- Spectral detectors such as multi-anode PMT (32 or more spectral “bins”) or CCD / array type detectors (hundreds of spectral “bins”)
  - Multi-anode PMT is becoming common on commercial confocal microscopes
  - CCD detector was common on ABI 4-5 color sequencing instruments, also has been used for multi-color LIF detection on chips.
  - With a large number of spectral bins (>32, ideally >100) – spectral deconvolution techniques can resolve many overlapping fluorophores simultaneously, and even resolve fluorophores from background fluorescence
  - Drawback: signals are split among many active detection elements, each of which has some inherent noise, so S/N may suffer
    - Cooling the detector can help with “dark” noise but a cooled CCD will still experience “readout” noise
  - CCD requires a bit of programming skill to work with, vs a PMT (simple analog current or voltage output for each channel).

Other detection options

- Photon counting
  - Both PMT and APD can be used in photon counting mode; at low signal level this can help discriminate bursts of photons from a “real” detection event from noise.
  - Time-correlated photon counting can be used for fluorescence correlation spectroscopy in combination with chip electrophoresis.
- Pulsed laser techniques
  - A pulsed laser and time-correlated photon counting can be used to extract fluorescence lifetime information, allowing discrimination of spectally overlapping fluors, or observing fluors at low overall concentration
  - Time gated detection can be used to “gate out” fast processes (*e.g.* Raman scatter) or slow processes (*e.g.* long-lived cellular autofluorescence), but need to consider detector response time
    - PMT is generally faster than APD in photon counting mode.
  - Intensified CCD can potentially be used for time gating and spectral detection
- Not for novices, and probably not for compact or portable instrumentation

Compact LIF detector for portable instrumentation



Configured here for capillary LIF;
Readily converted for chip use by addition of alignment pins registered to holes on chip manifold

Compact detectors can be prototyped using commercially available optics and mounts for ½" or smaller, but some optical elements may be hard to find.

www.linos.com

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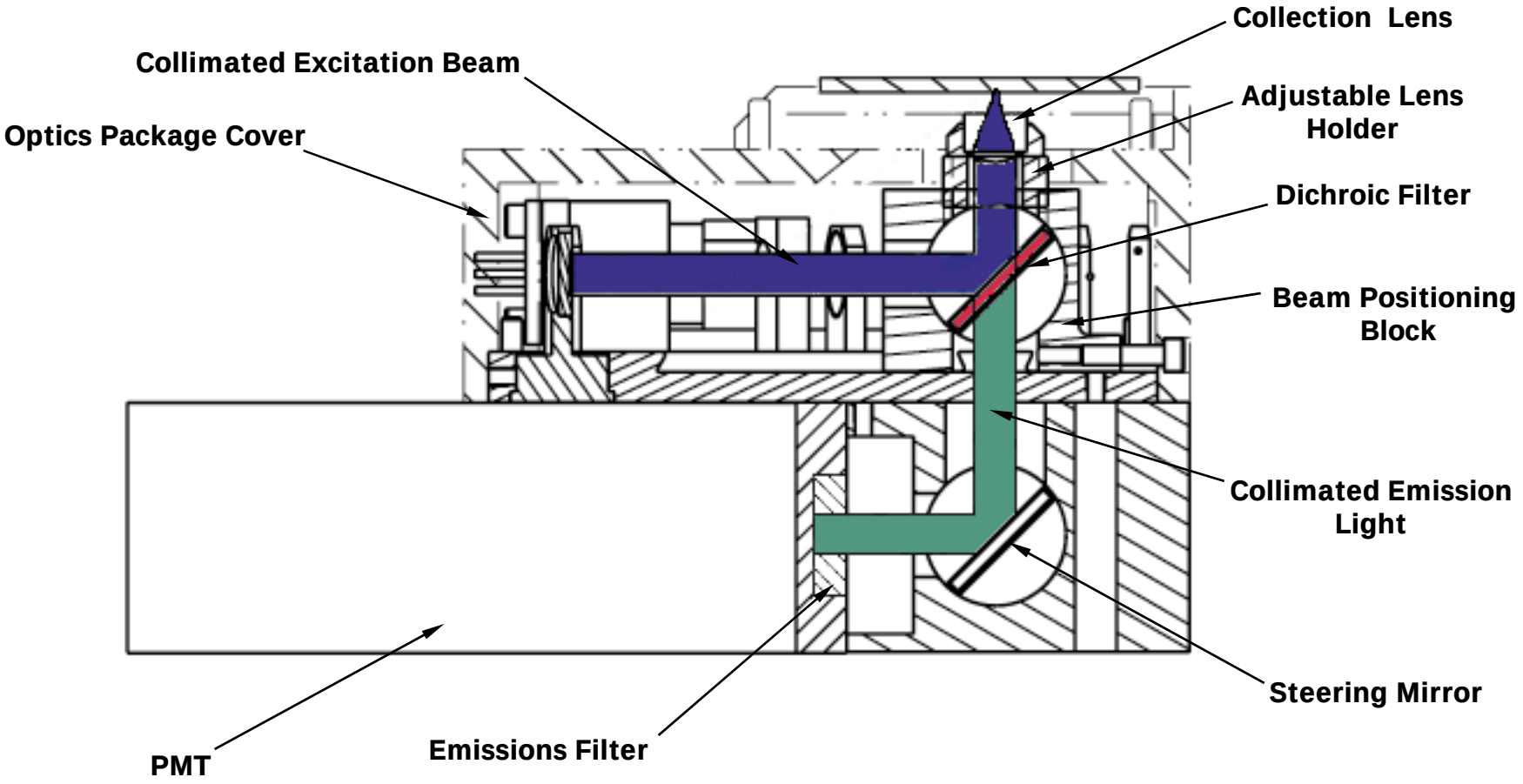
Gen2 LIF Optical System
Epi-Fluorescent Configuration



Approximate size: 3.25” long x 2.25” wide x 1.3” tall

Renzi 3/18/02

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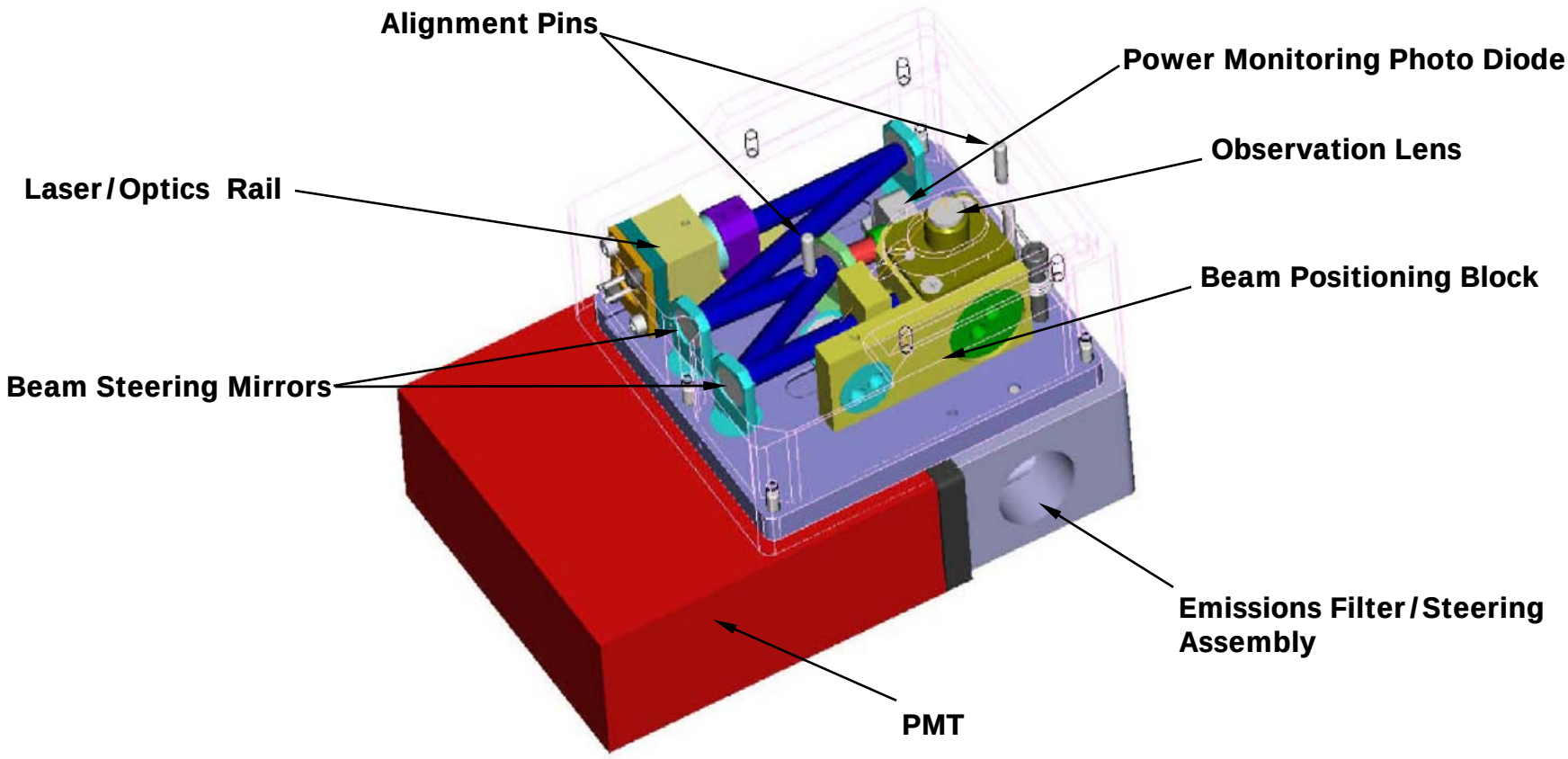
Gen2 LIF Optical System



Approximate size: 3.25” long x 2.25” wide x 1.3” tall

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