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Microfluidic Laminar-Flow Extraction of Functional Proteins From Cell Lysate

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Sandia National Laboratories
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Sandia is a multiprogram laboratory operated by Sandia Corporation, a Lockheed Martin Company, for the United States Department of Energy's National Nuclear Security Administration under contract DE-AC04-94AL85000.

Extraction Flow Configuration

PEG-rich feed
35% PEG, 1.5% salt

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

$$\frac{\text{salt-rich feed}}{\text{PEG-rich feed}} \sim 6 - 8$$

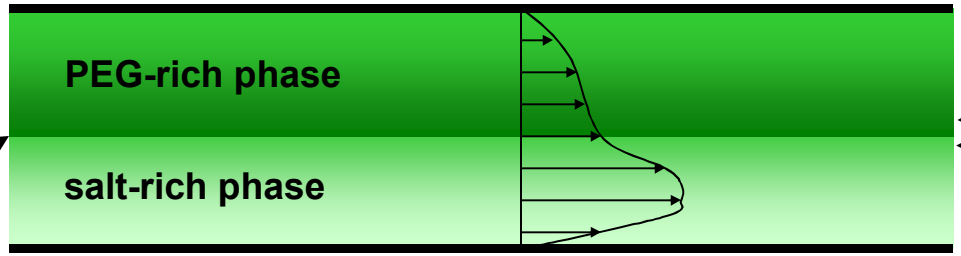
Aqueous sample

Sample diffuses into both phases with partition coefficient *K*

salt-rich feed
16% salt

Typically large Peclet number (~1000)

Far downstream…



stable interface

Equilibrium at interface

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

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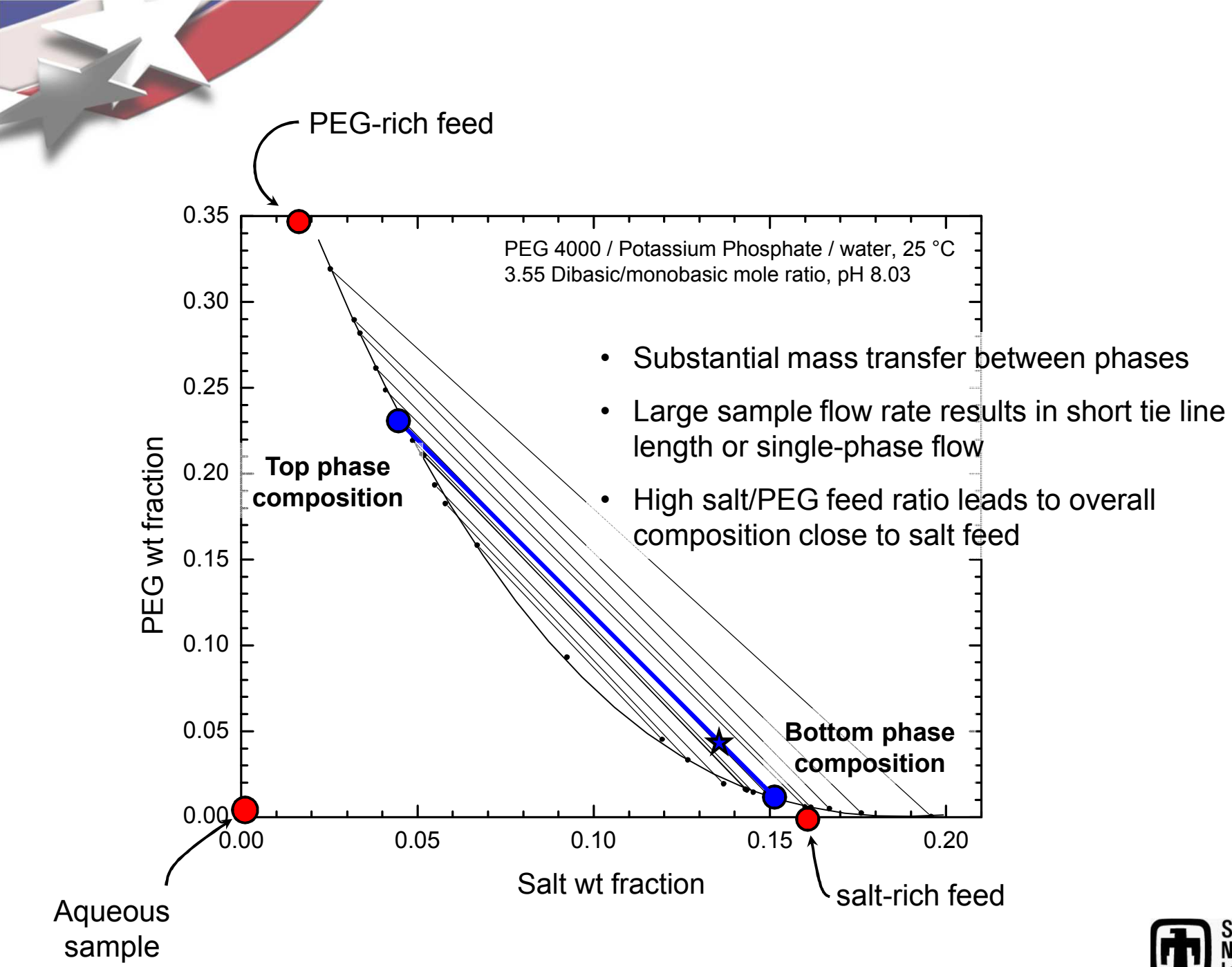

- Substantial mass transfer between phases
- Large sample flow rate results in short tie line length or single-phase flow
- High salt/PEG feed ratio leads to overall composition close to salt feed

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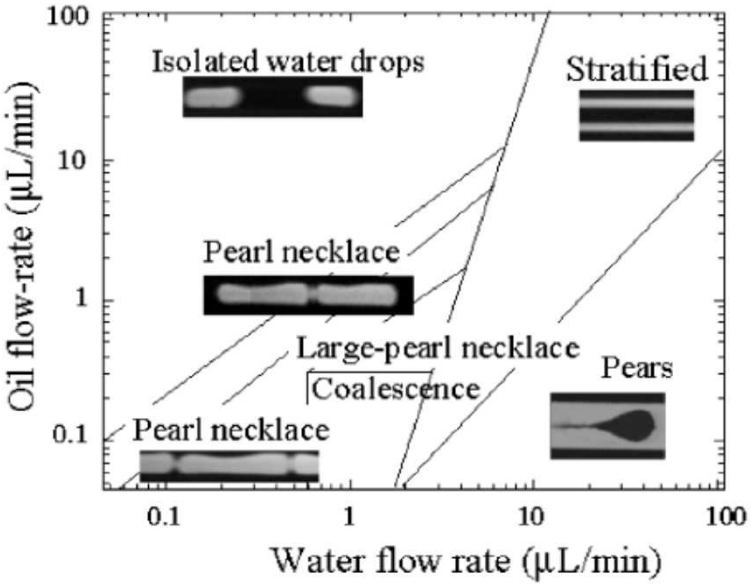
Extraction in microfluidic chips

- There are several reports of aqueous/organic extraction and stratified flow in microfluidic chips
  - In oil/water systems, the pure components are nearly immiscible in each other (very small single-phase region)
  - *Ca* = $\mu U / \gamma$ is important parameter for determining flow characteristics
  - High interfacial tension (~10-50 mN/m) leads to complex flow behavior
  - 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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Aqueous Two-Phase Systems in Microfluidic Chips

- Very low interfacial tension (1-100 μN/m) makes stratified flow easy to achieve
  - Regimes with droplets can occur
- A few examples in the literature of ATPS in microfluidic devices for protein or cell fractionation

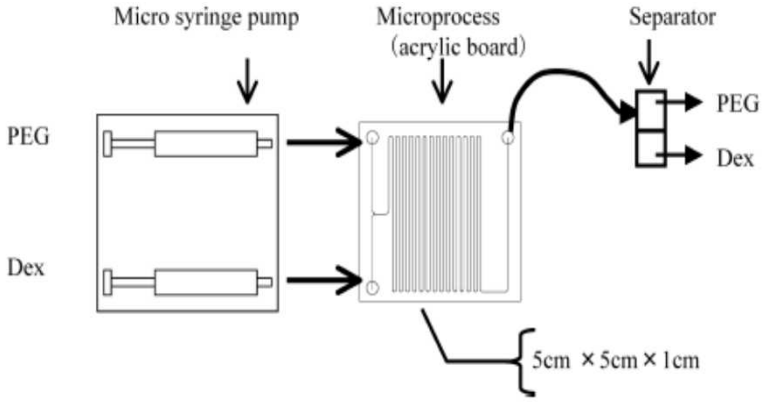
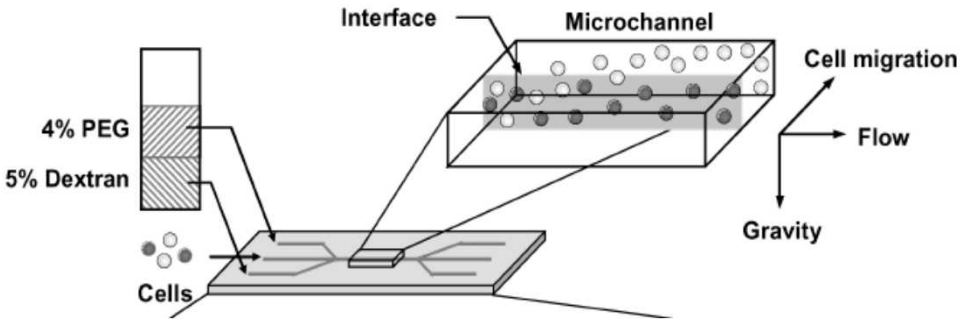


Fig. 2 Whole image of microflow aqueous polymer two-phase system

Asami *et al.*, *Kagaka Kogaku Runbunshu* 2004



Nam *et al.*, *Biomed. Microdev.* 2005
(Fractionation of live/dead cells)

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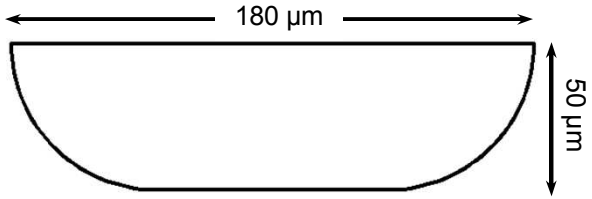
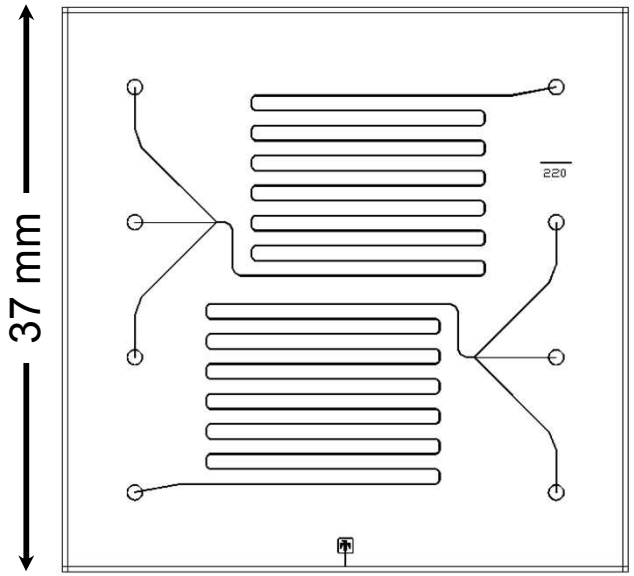
Initial channel designs

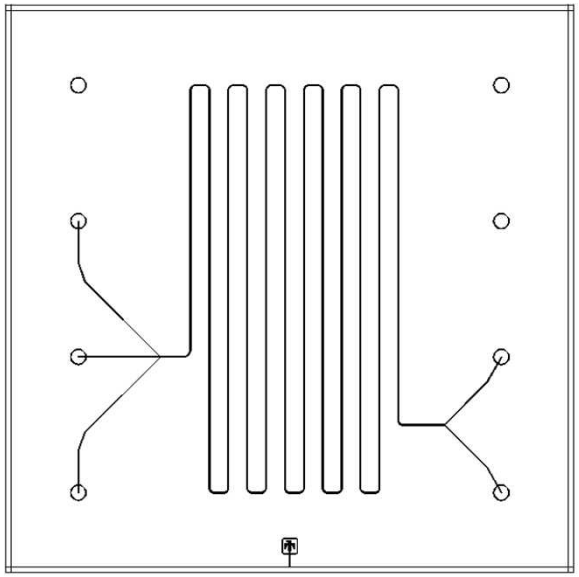
- Glass chips for initial studies
- Long, serpentine channels for long residence time in small footprint
- Relatively large diameter keeps pressure drop manageable
- Channels are covalently coated with polyacrylamide or poly-*N*-hydroxyethylacrylamide to prevent protein adsorption and unusual flow patterns

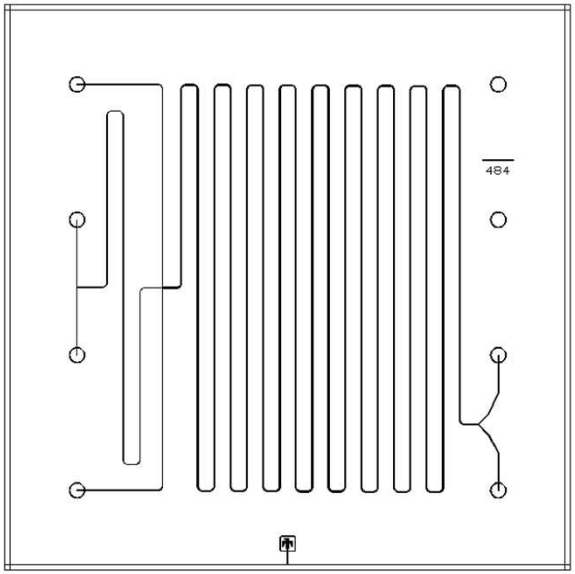


Channel cross section



220 mm channel


326 mm channel



484 mm channel

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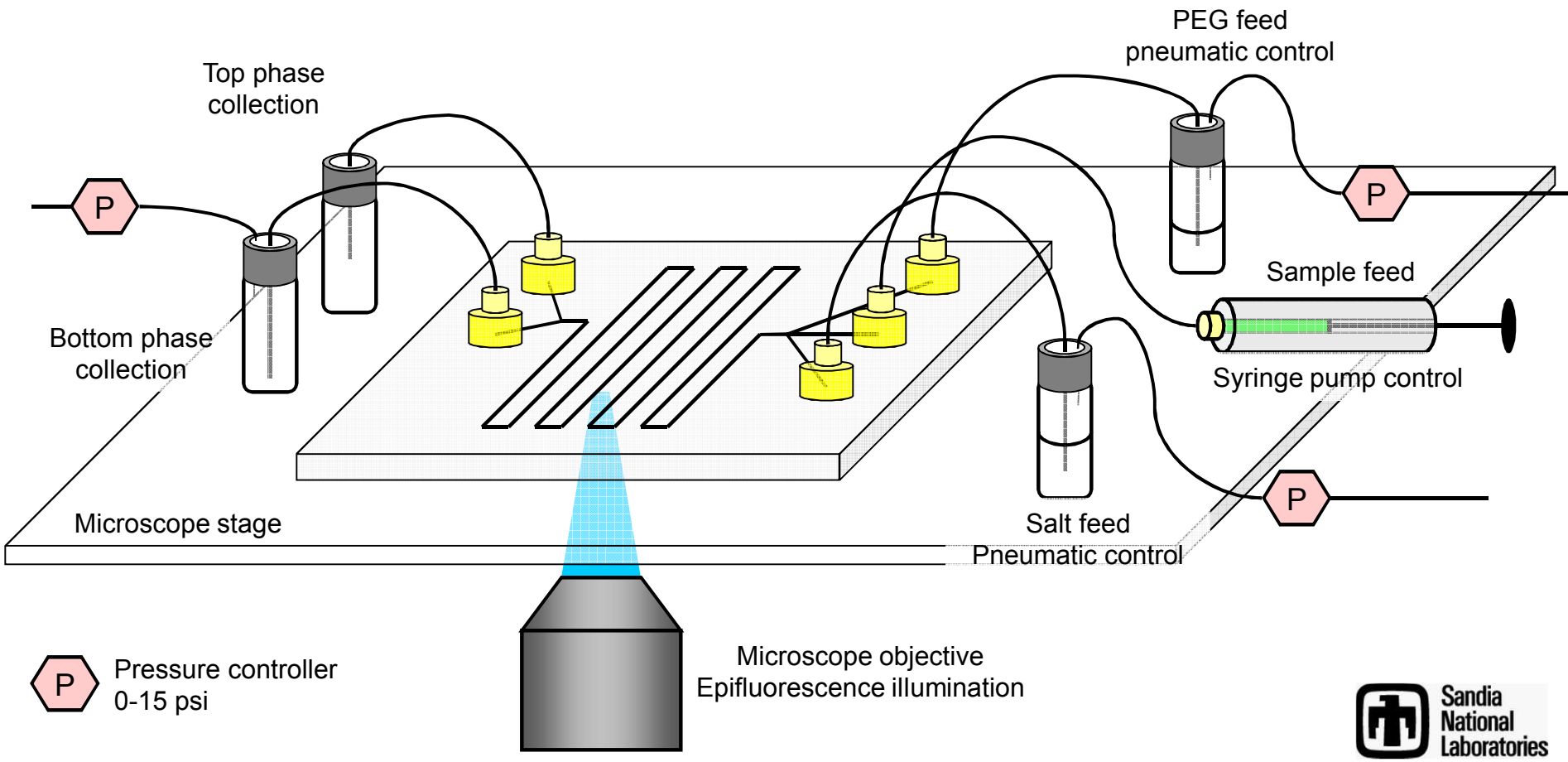
Experimental Setup

One possible configuration – other setups are possible!



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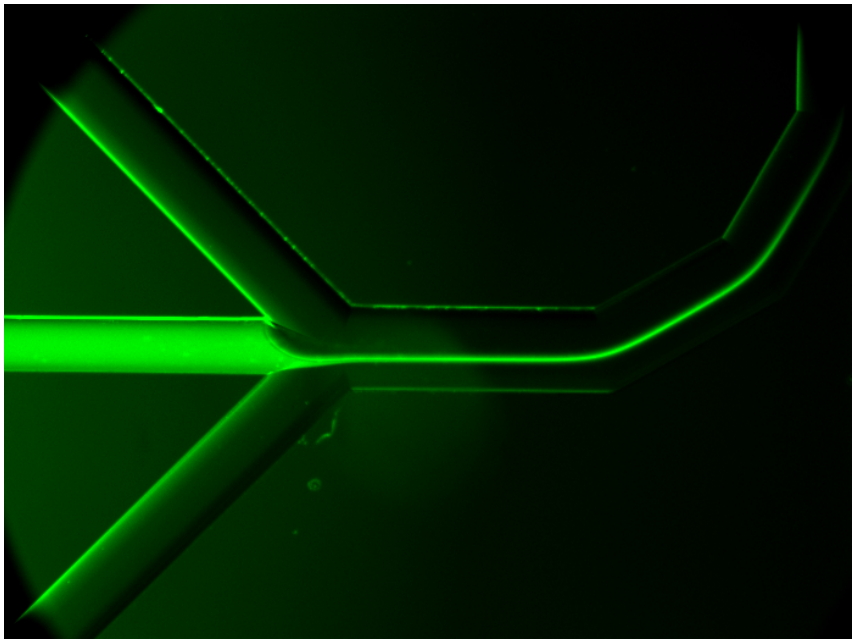
Stability of interface

- Interface between phases remains stable for entire length of channel (>200 mm)
  - Linear velocities of 5-20 mm/s tested
  - Video shows hydrodynamic focusing of FITC sample, with partition to PEG-rich phase along length of channel.

35% PEG
1.5% salt



FITC in PBS

16% Salt

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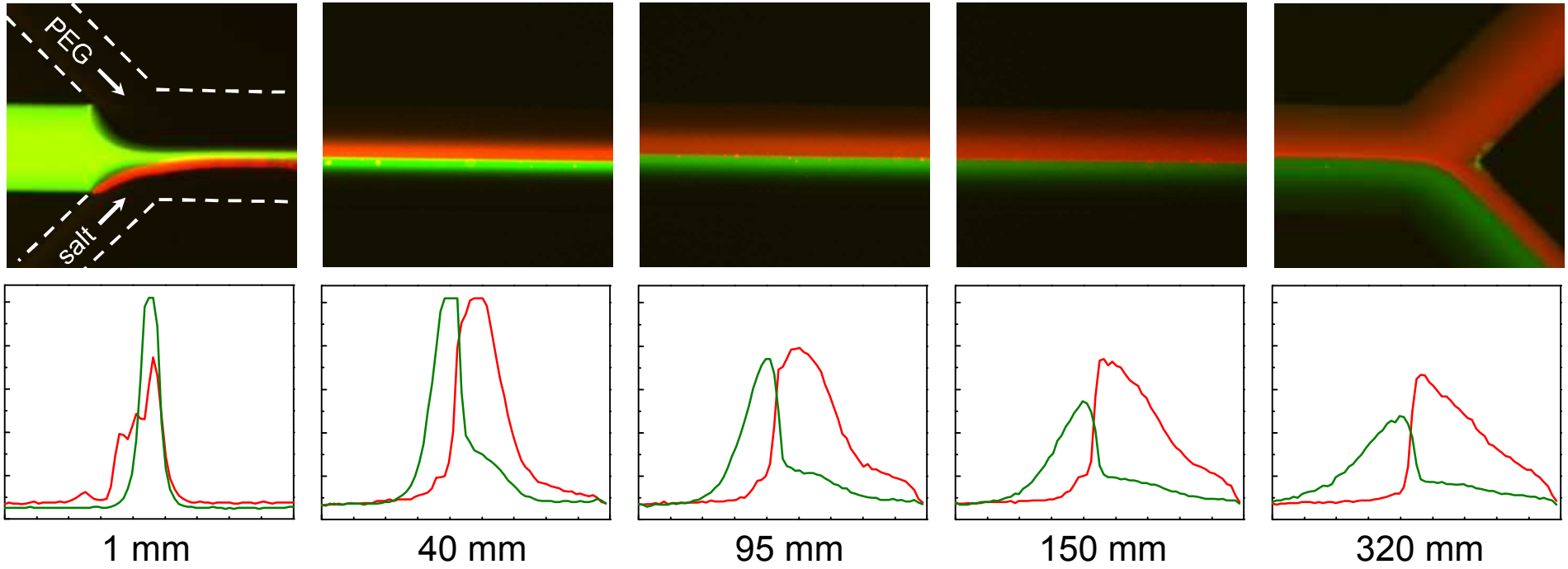
Partitioning of Proteins

- 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 phase; β–gal partitions strongly to PEG



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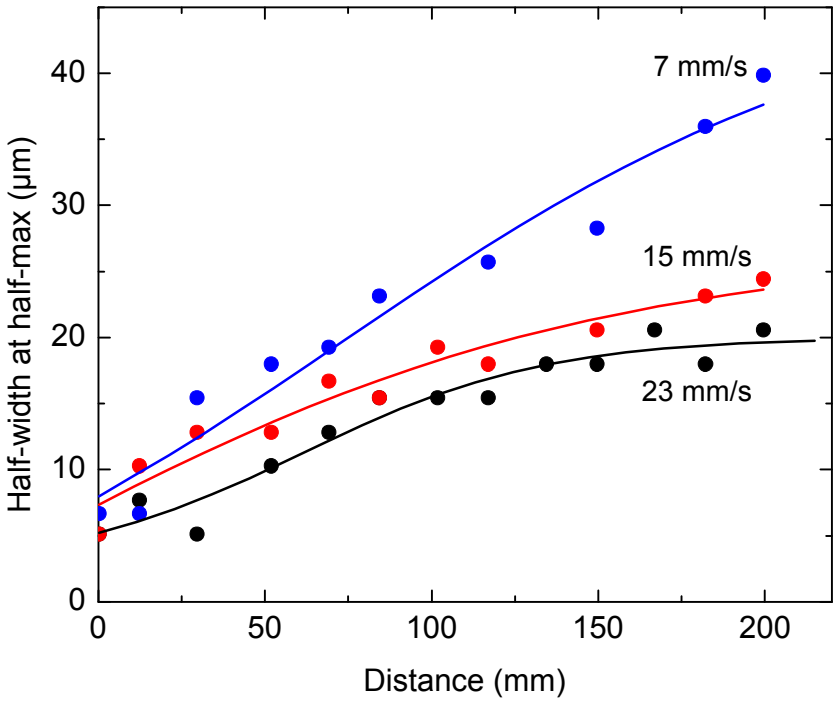
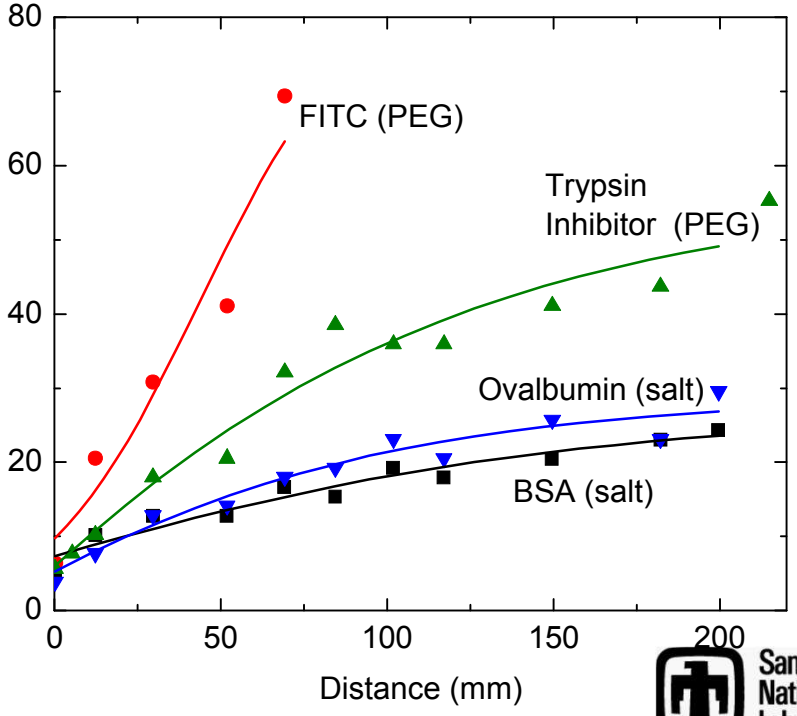
Spreading of sample from interface

- Equilibration of sample concentration across the channel depends on flow rate and diffusivity of analyte
- Flow rate is smaller on PEG side of interface, leading to longer residence time and greater spreading of analytes
- Diffusivity may be different in PEG and salt phases

Spreading of BSA on salt-rich side as a function of velocity



Spreading of different analytes at 15 mm/s



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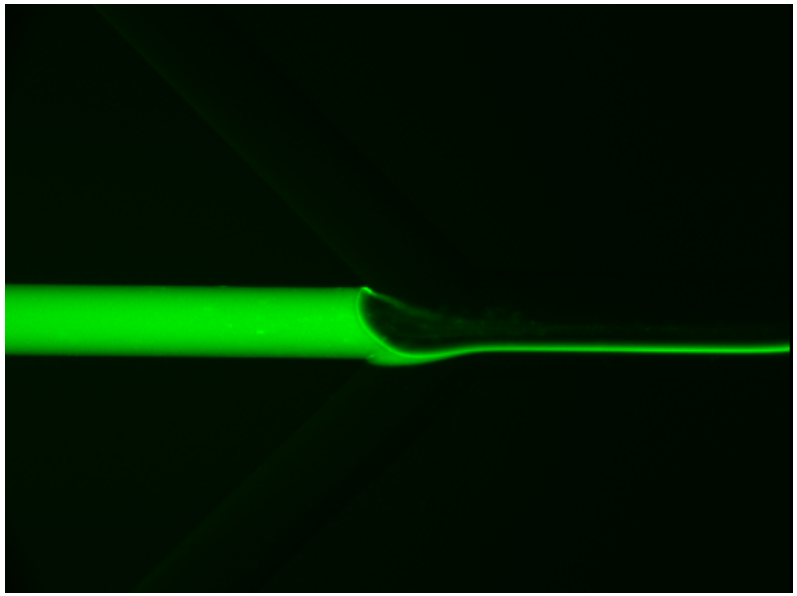
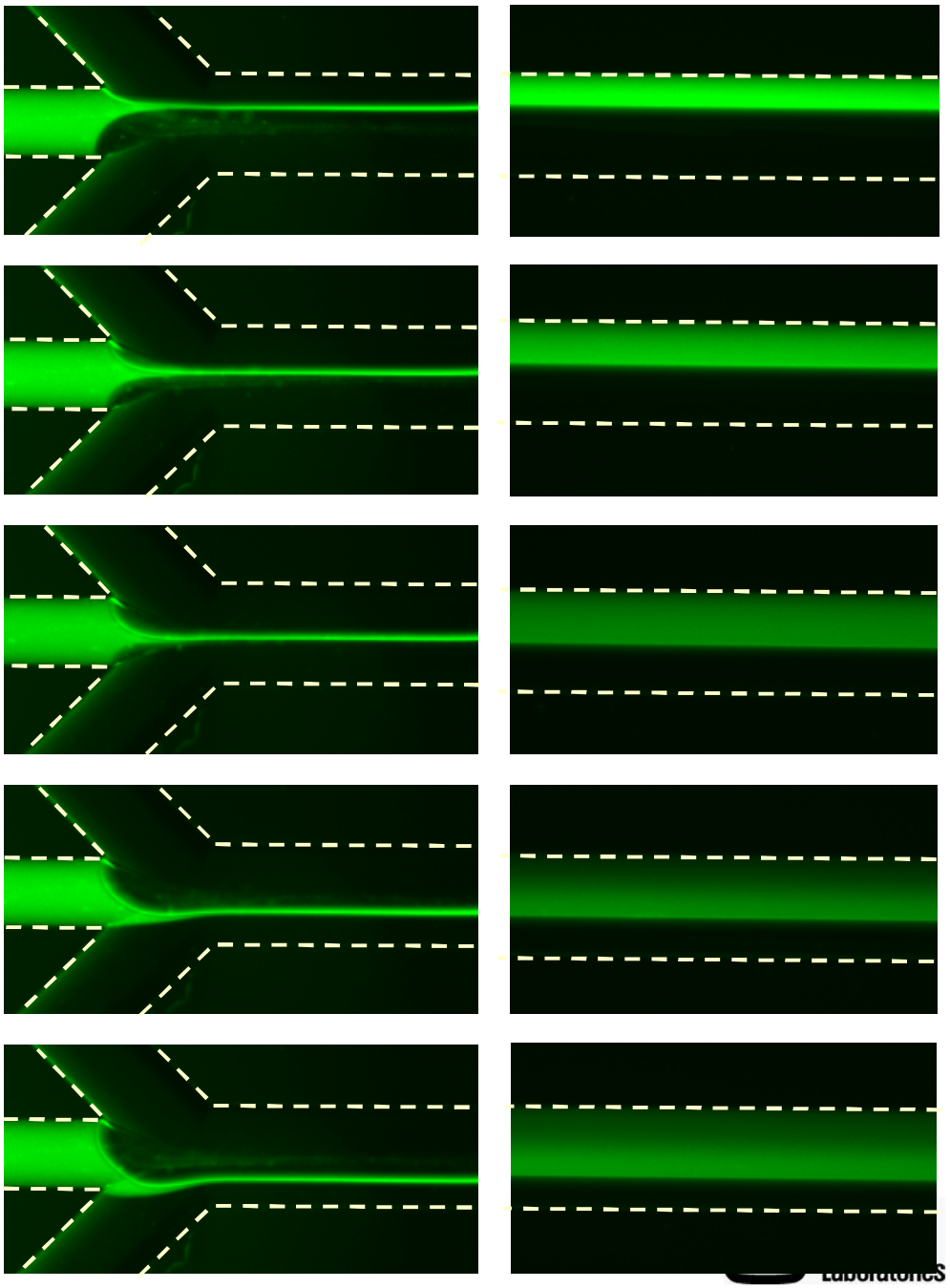
- Feed ratio of PEG and salt determines position of the interface
- Volume ratio of phases affects equilibrium amount of sample recovered in each phase
- For interface near center of channel, PEG/salt ~ 1/7

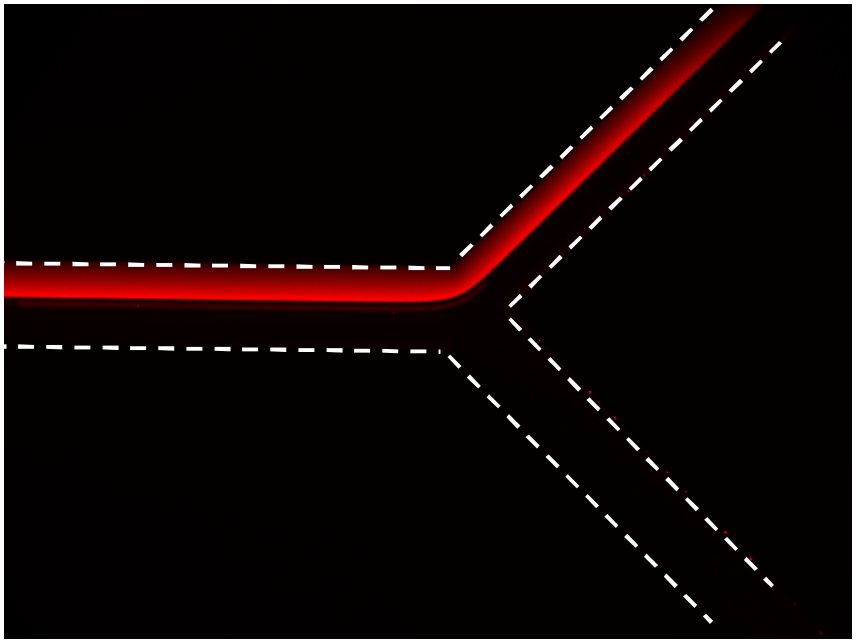




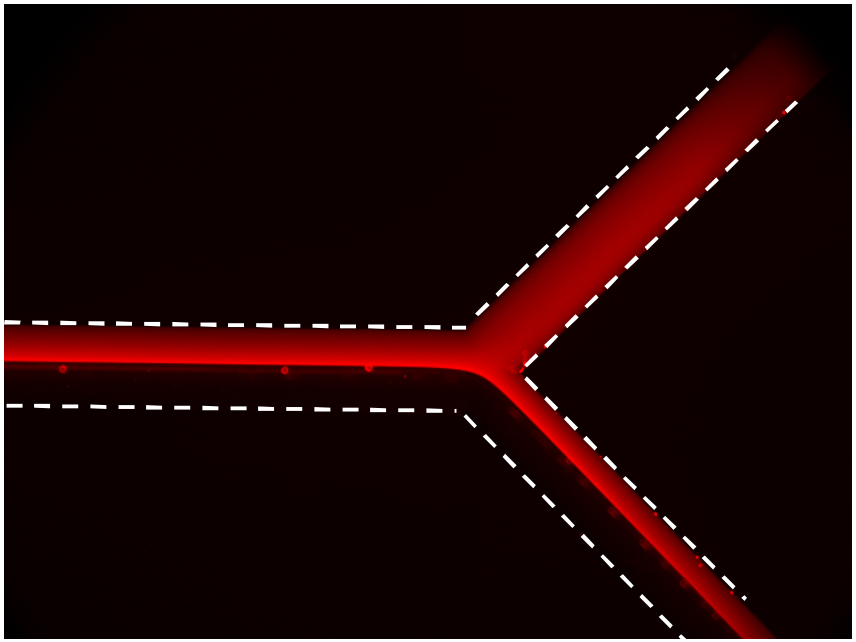
Splitting flow at outlet

- Apply a differential pressure to one outlet for precise shifting of interface
- Allows collection of desired phase with minimal contamination
- Maximal recovery of desired component requires:
  - Spreading of desired component away from interface
  - Precise split between phases, minimizing loss of desired phase

Pressure ↑



Pressure ↓



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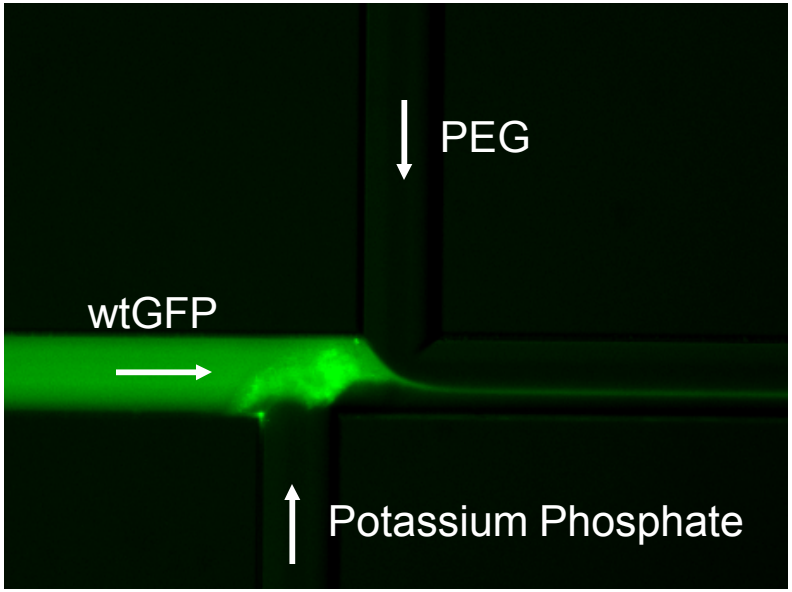
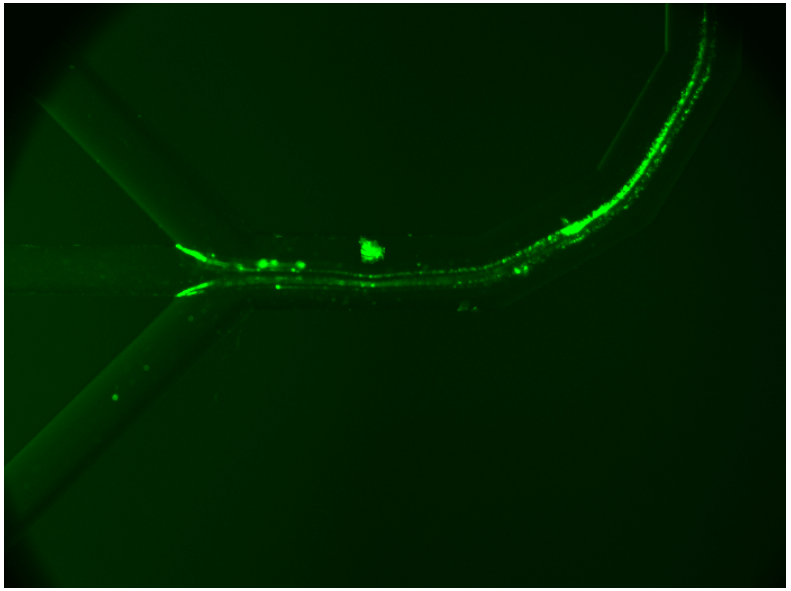
- Not all proteins are compatible with the high PEG and/or salt content
- Proteins may tolerate <15% or >50% PEG
- Addition of 0.01-0.1% Tween 20 can reduce aggregation and precipitation, although detergents can affect the phase equilibrium.



GFP precipitates with high salt concentration (Tween 20 helps prevent this)



Concavalin A precipitates badly in both PEG and salt even with Tween

[1]Albertsson, *Partition of Cells & Macromolecules*, 3rd ed, 1986

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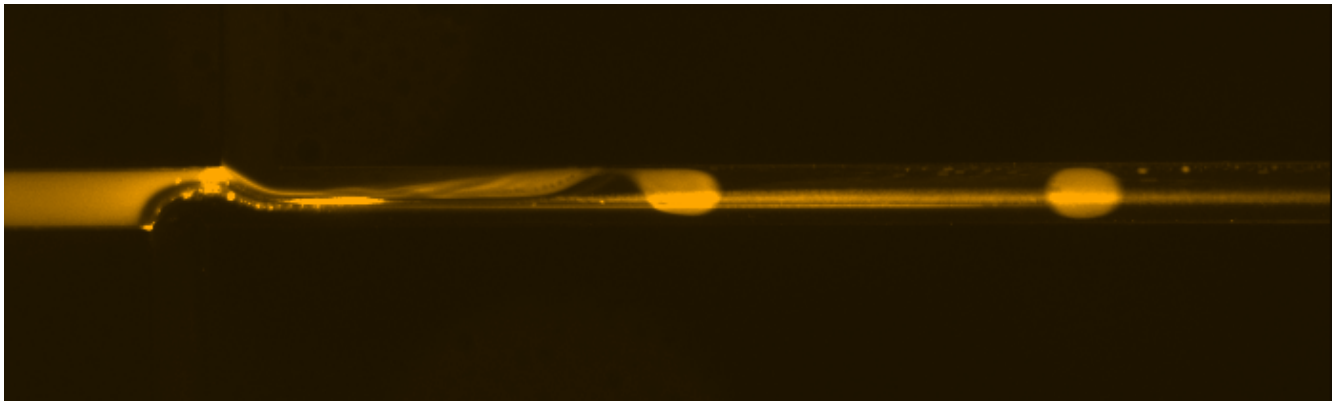
Segmented flow

- Droplets or segmented flow can occur, with alternating slugs of PEG-rich and salt-rich phases
- Rapid equilibrium between two phases – long channels not required
- Interesting phenomenon, but separation of phases at outlet is difficult with a static, continuous flow device



Segmented flow with β-gal at low flow rate (~.4 psi ΔP, <1 mm/s) following precipitation at intersection

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Recovery of functional enzyme: β-galactosidase

- ATPS is supposed to be mild enough to allow recovery of structurally intact, functional proteins
- We tested β-galactosidase activity in "Top" and "Bottom" outlets from chip extraction experiment
  - Used conversion of fluorogenic substrate to measure activity

PEG/salt partition coefficient (activity measurement)		Recovery fraction of enzyme activity in top outlet
Off-chip	On-chip	
106	6 – 31	55 - 78%

- Effective on-chip partition coefficient and recovery fraction depend on how much of the top stream is sent out with waste to the bottom outlet (operator-dependent!)
- Activity measurements have big error bars

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Engineering proteins to modify partitioning

- Strategy 1: create fusion proteins with β-galactosidase
  - β-gal has very strong preference for PEG phase
  - Limited success with expression of GFP-β-gal fusion
  - β-gal is a huge protein to use as a fusion partner!
- Strategy 2: employ small peptide fusion tags to modify partitioning
  - Kohler *et al*, *Nature Biotech* 1991 report (AWWP) sequence
  - Later researchers (Fexby *et al*) report $(WP)_4$ and Y_3P_2, among other sequences
  - Key seems to be combination of aromatic amino acids (especially W and Y), and proline (helps to keep terminus near surface of protein)

Case 1: GFP

- Variant used was AcGFP1 (Clontech's latest "enhanced" monomeric GFP)
  - His_6-AcGFP1 (purchased)
  - His_6-Y_3P_2-AcGFP1 (cloned & expressed in-house)
  - His_6-$(WP)_4$-GFP1 (cloned & expressed in-house)
- All variants (including wt) have issues with precipitation; $(WP)_4$ version may be less problematic

Variant	PEG/salt Partition Coefficient		Recovery in "Top" stream from chip*
	Off-chip	On-chip	
H_6-AcGFP1	1.5	1.5 - 2.0	18%
H_6-Y_3P_2-AcGFP1	13	9 - 13	62%
H_6-$(WP)_4$-AcGFP1	34	27-37	81%

- Significant uncertainties in outlet flow rates!
- Total fluorescence out is similar to total fluorescence in, ±20%
- ATPS process does not seem to degrade GFP fluorescence

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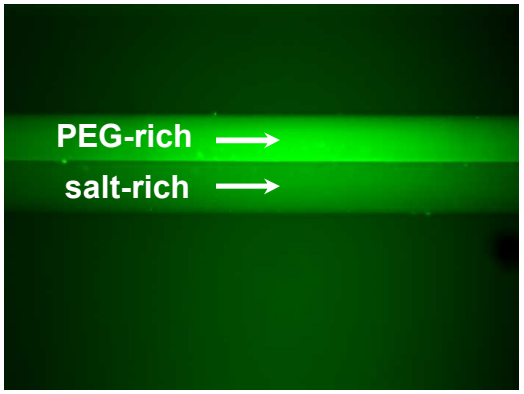
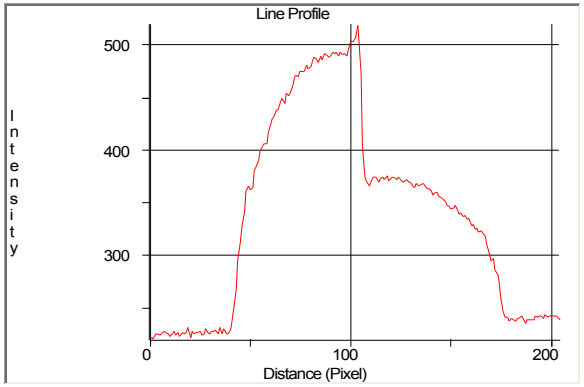
Fluorescence micrograph near channel end

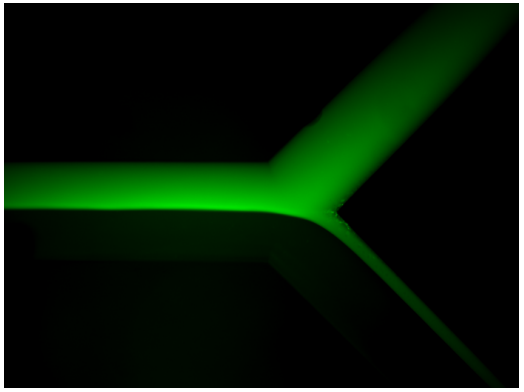
Line profile of fluorescence intensity across channel

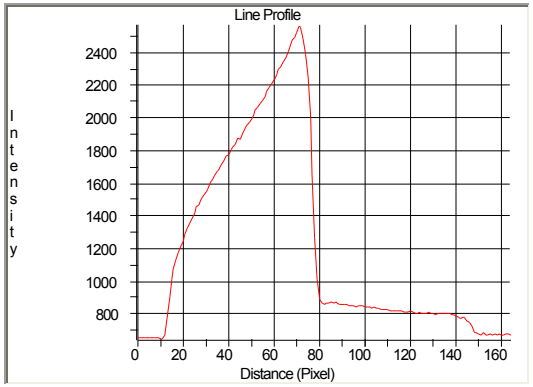
"Wild type" His_6-AcGFP1



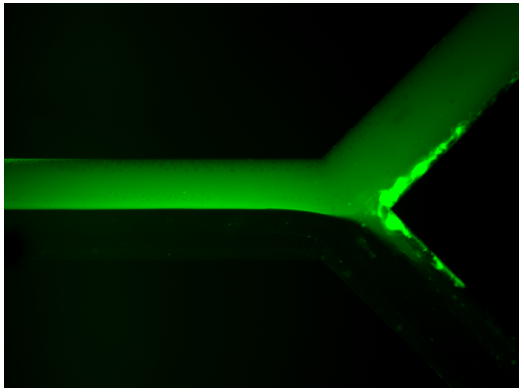


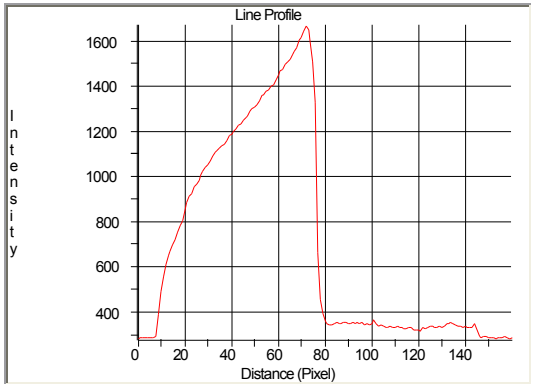
$H_6Y_3P_2$AcGFP1





$H_6(WP)_4$AcGFP1





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Case 2: GST

- Glutathione-S-Transferase – enzyme which is commonly used itself as a fusion tag for purification
- We have produced a “wild-type” and a version with an *N*-terminal $(WP)_4$ tag
- Activity is measured by a colorimetric assay (low sensitivity)
- Just yesterday obtained a cell lysate of H_6-$(WP)_4$-GST-Lumio with measurable GST activity in soluble fraction
- Partition coefficient (off-chip) measured by GST activity assay is ~40 for $(WP)_4$-tagged variant
- Chip experiments with wild-type and tagged GST haven’t happened yet!

Partitioning of other cell components

- DNA – performed measurements with commercial *E. Coli* genomic DNA, assayed using PicoGreen (fluorescent dsDNA stain).
  - **Off-chip**: partition coefficient ~ 0.11 (prefers salt phase)
  - **On-chip**: probably stays near interface due to low diffusivity. Recovery in top stream ~ 0.2%
- Cell debris
  - **Off-chip**: seems to partition to salt, but may simply be a result of density difference and centrifugation
  - **On-chip**: large material should stay near interface. This material may also stick to channel walls, get stuck in dead volumes (at inlet), and get stuck at stagnation point (at point where outlets split). I haven't been able to observe anything conclusive, e.g. by using fluorescently labeled lipids.
- Total protein content – not sure! Nano-orange protein assay is severely compromised by the high salt content. Currently waiting for shipment of EZQ protein kit, which is insensitive to contaminants

Other enzymes to test

- Enzymes with fluorogenic substrates are easiest to quantitate given inherently small amounts used in chip experiments
- Proteases
  - I measured thrombin parititioning by activity (K~0.1) – could be a candidate for tagging, but recombinant thrombin has to be expressed as a proenzyme
  - Caspases could be an interesting target (relevant to apoptosis, active forms can be expressed directly in E. Coli, fluorogenic test substrates are available)
- Numerous other reporter enzymes (glucorinidase, peroxidase, phosphatase, etc) have fluorogenic substrates