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FISH ‘n’ Chips – A microfluidic processor for Isolating and Analyzing Microbes

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Joint Genome Institute (Philip Hugenholtz)

Project Start Date: 8/18/2009
Project End Date: 8/17/20012

Project Overview

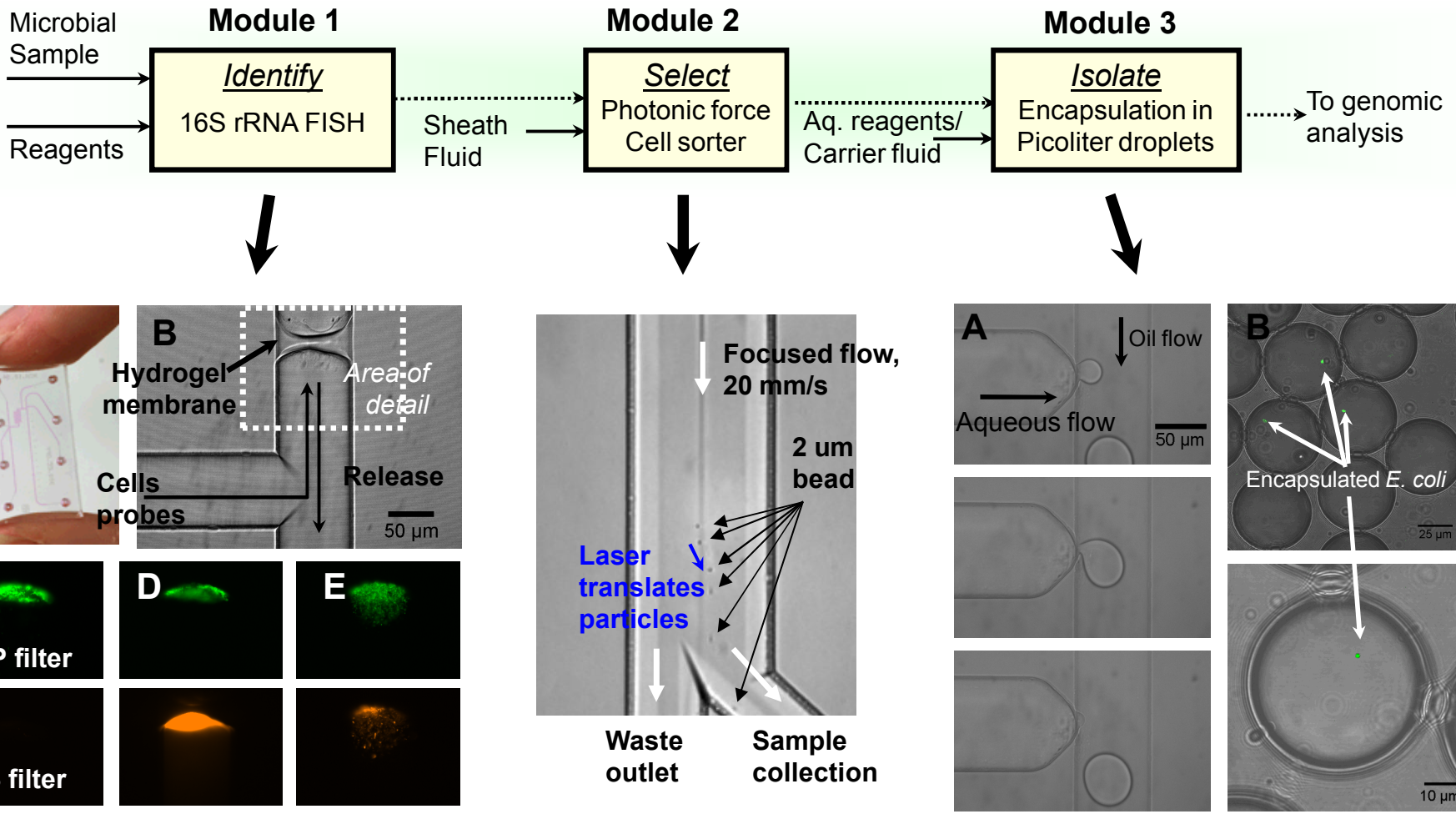
- The majority of microbes can not be cultivated by known methods, but are nonetheless likely to play a significant role in the ecology at all sites in the body, and thus it is critical to develop techniques to obtain samples of microorganisms for genetic analysis, without relying on traditional culture-based techniques.

- Develop technology to recover genomic DNA, suitable for sequencing, from individual (single cell) specimens of rare, previously unknown, and/or uncultivable microbes from the oral cavity.
  - Our role in this project is mostly technology development
  - Our collaborators at NYU have an interest in the role of bacterial biofilms in inflammatory processes, and possible roles bacteria may play in the progression of pre-cancerous lesions to oral cancer
  - We are also involved in a DOE-funded project to study bacterial communities in environmental water samples (*e.g.* Hanford) , and we are developing similar technical approaches for this work.

Motivations

- Metagenomic studies have revealed far greater diversity of bacteria than was previously realized, mostly because previous studies relied on culturing techniques, and missed the large majority of bacteria that don't grow easily in the laboratory.
- Much of the diversity is inferred only from bits of ribosomal RNA sequence uncovered in shotgun metagenomics studies
  - 16S sequence reveals phylogenetic relationships, but says nothing about function.
- a good way to understand bacterial function is from genomic sequence information. The "traditional" approach is to grow up a pure culture (clonal population) of a particular bug, extract genomic DNA, and sequence. However – the large majority of microbes can not be grown in pure culture by known laboratory techniques, for a variety of reasons.
- So we can either work on new techniques for culturing difficult-to-culture bugs, or develop culture-independent techniques for DNA sequencing.
- This entire field is made possible by recent developments in DNA sequencing ("next-generation" or "ultra high-throughput" sequencing techniques)

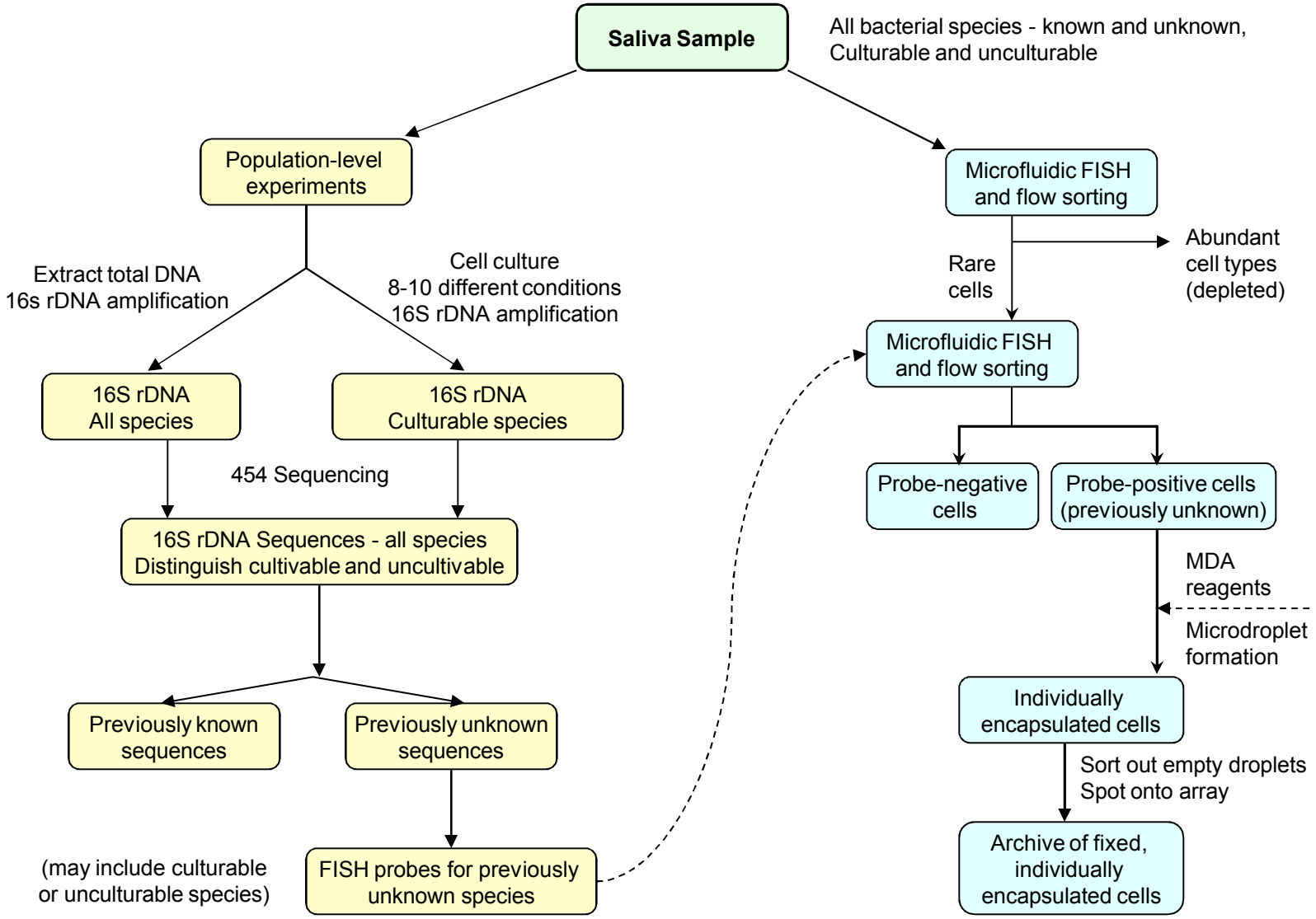
“FISH-n-Chips” Microfluidic Processor



FISH at a photopatterned membrane on a chip

Optical deflection of selected particles into a collection channel

Microdroplets as picoliter-volume reactors for single-cell genome amplification



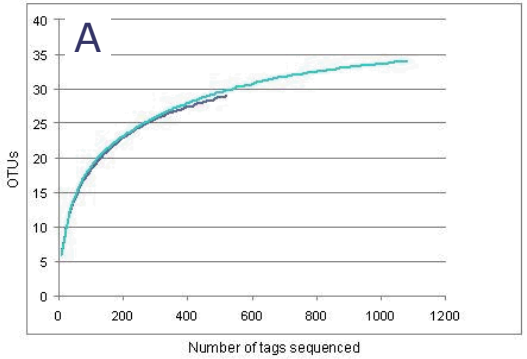
Objective: Detection of unknown, uncultivable bacteria sequences in saliva based on 16S rRNA sequence; Design of FISH probes for unknown uncultivables
Methods: Sequencing, microbial cell culture, bioinformatics

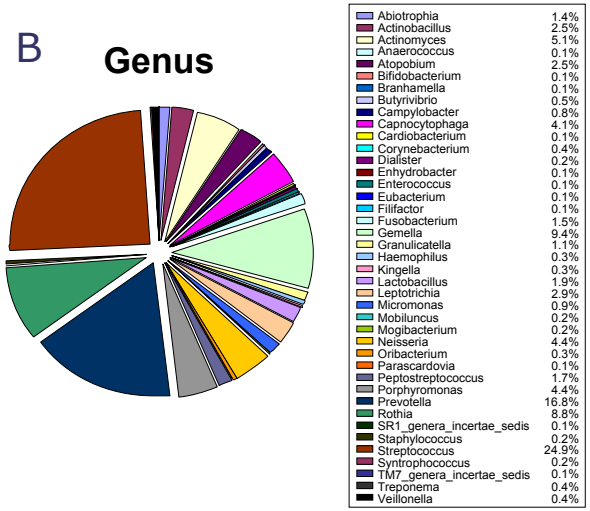
Objective: Use FISH to Isolate specimens of previously unknown bacteria; MDA for whole-genome sequencing.
Methods: Microfluidic device for on-chip FISH, optical flow sorting, and cell encapsulation.

Metegenomics of the oral microbiome (NYU)

the persistent presence of bacteria at the tumor sites in oral cavity raises intriguing questions about the role of bacteria in progression of oral squamous cell carcinoma (OSCC). We sequenced (by 454) ~26,000 PCR amplicons that span the V4-V5 hypervariable region of ribosomal RNAs from saliva samples of two OSCC patients.

Sample	Total Reads	Trimmed Tags	Unique Tags	OTUs(unique)
S19	11,481	11,141	5,837	443
S21	15,170	14,831	7.351	587





Our initial study provides numerous examples of previously undiscovered, low-abundance phylotypes. We are expanding our sequencing efforts of both OSCC and normal samples, with an aim of discovering relevant targets for further analysis.

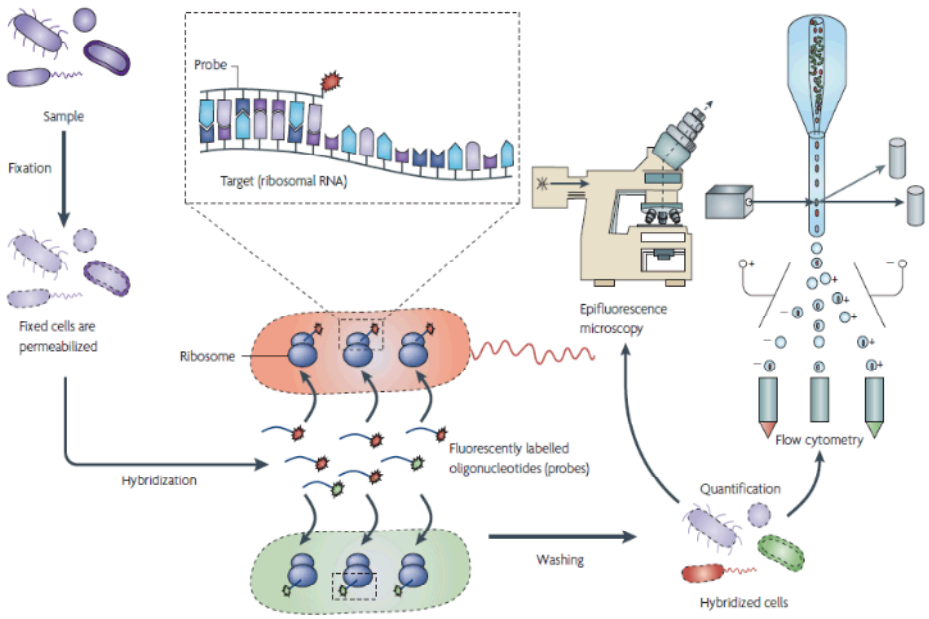
Newly discovered 16S sequences will be used to design FISH probes for identifying and selecting individual cells of interest from saliva samples. FISH or antibody-based sorting can also be used to deplete saliva samples of high-abundance species, to improve the chances of finding rare species. FISH probes need to distinguish among near neighbors *in saliva* but not necessarily from the entire bacterial domain.

FISH for bacterial identification

(*FISH = Fluorescent In Situ Hybridization*)

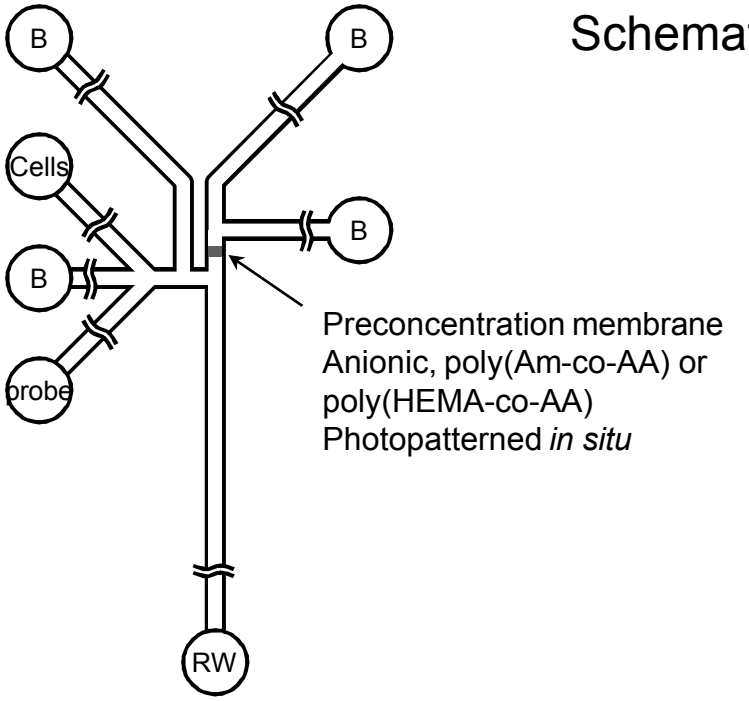
- Hybridize labeled oligonucleotide probe to RNA in intact, fixed cells.
  - Usually 16S rRNA to establish identity (can be thousands of copies per cell – good signal amplification).
  - Probing for mRNA is also possible, usually weaker signal.

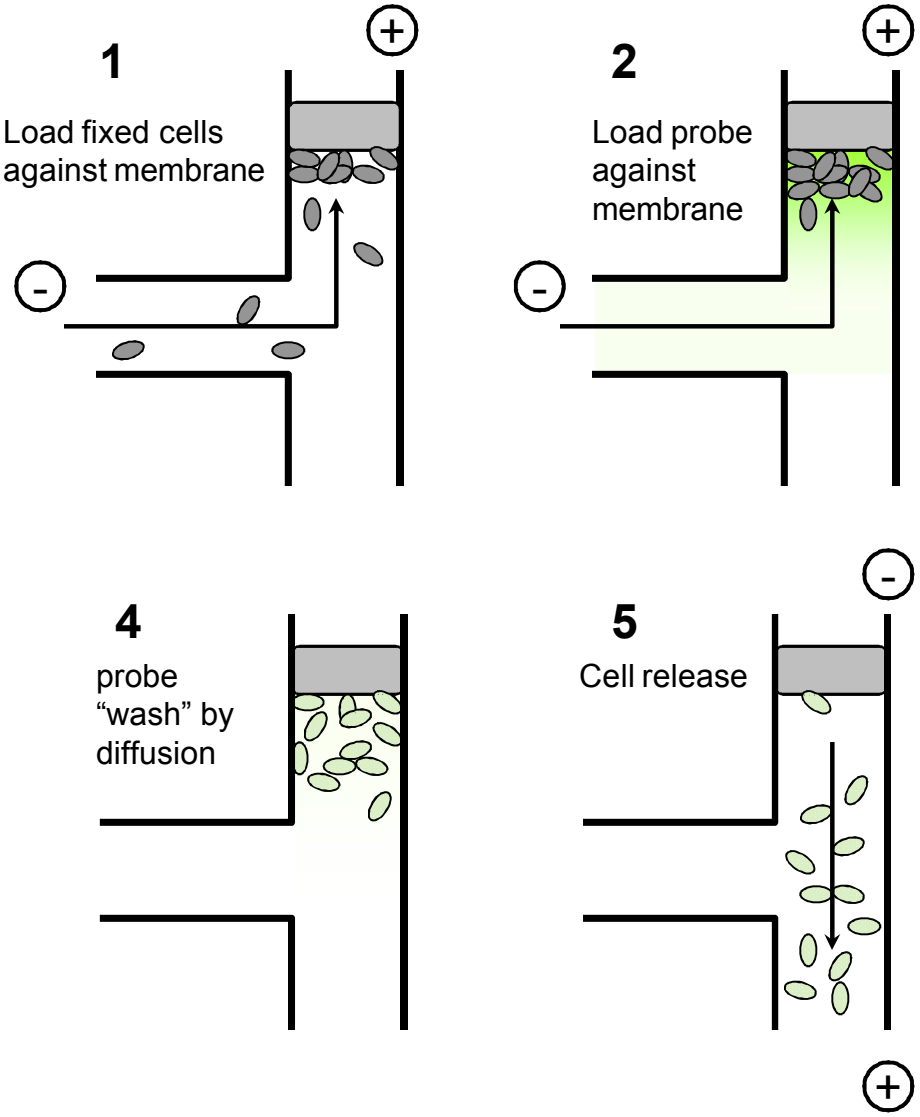
- Technique is robust, but somewhat labor-intensive
- Signals can be assigned to specific cells via imaging or flow cytometry
- A low degree of multiplexing is possible (1-3 probes plus a DNA stain like DAPI)
- Drawbacks: signal can be weak for environmental samples; can have problems with sample loss or difficulty finding cells in samples with low cell density.



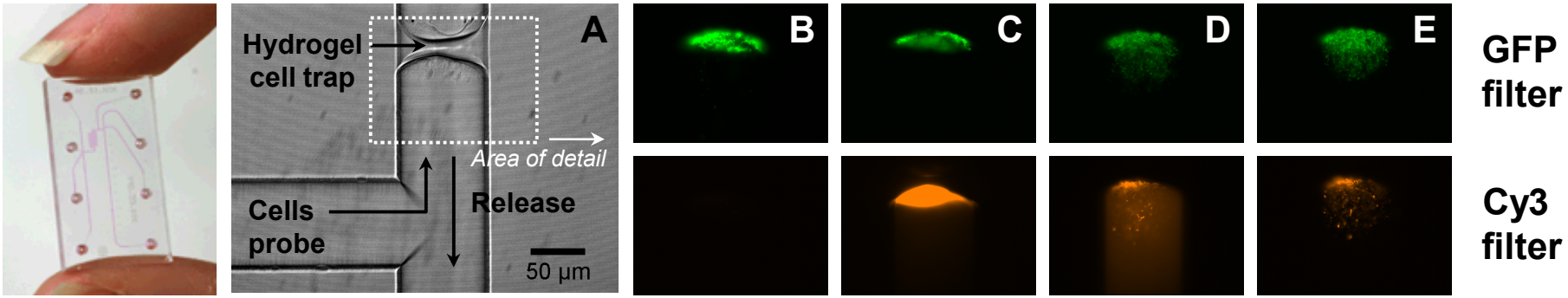
Amman and Fuchs, Nat. Rev. Microbiol. 2008

Schematic illustration of FISH at charged membrane





Example – GFP-expressing *E. coli*

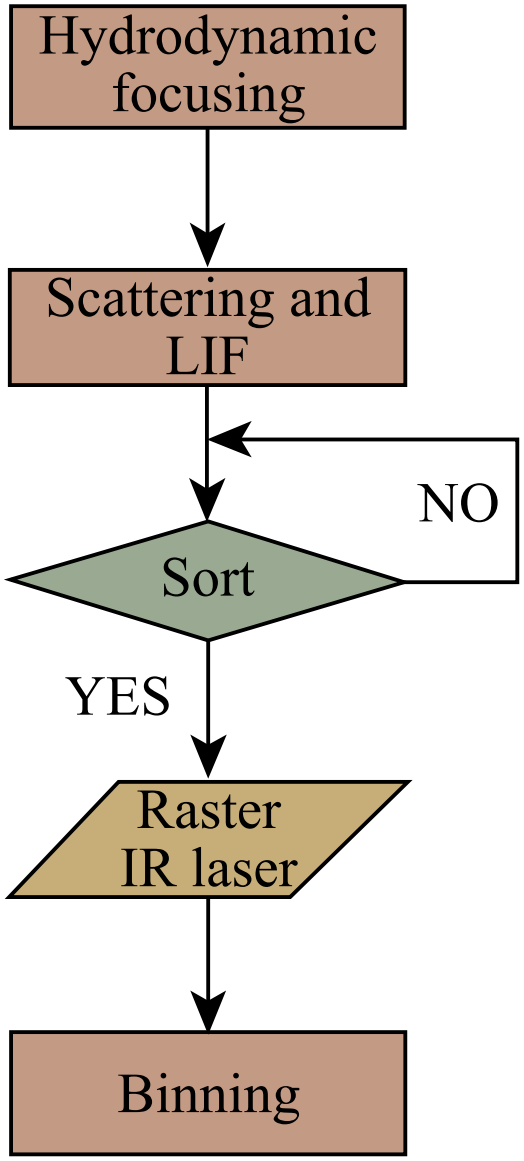


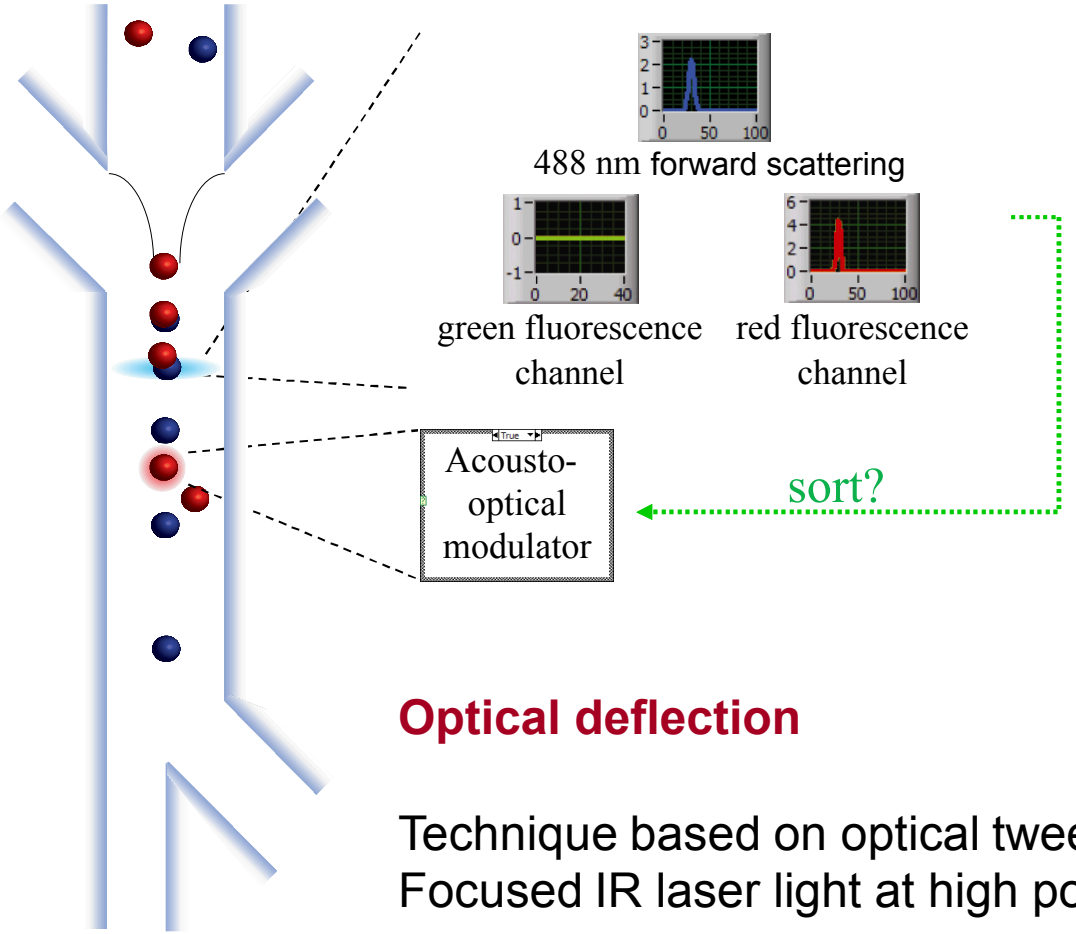
A. Brightfield image of microchannel
B. Concentration of GFP-*E. coli* at membrane
C. Concentration of Cy3-labeled probe at membrane
D. and E. Washing away probe by diffusion, leaving behind cells now showing Cy3 fluorescence

Chip FISH experiments

	E. coli	*L. acidophilus*	*S. mutans*
NON338	weak	-	-
Eco681	+		
LAB158	+	+	
MUT590			+

Optical μFACS - Principle





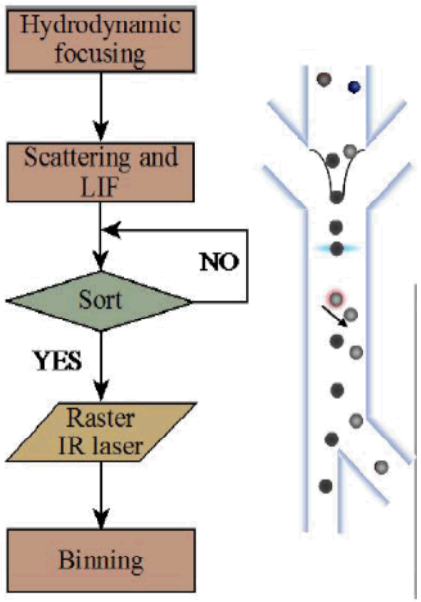
Optical deflection

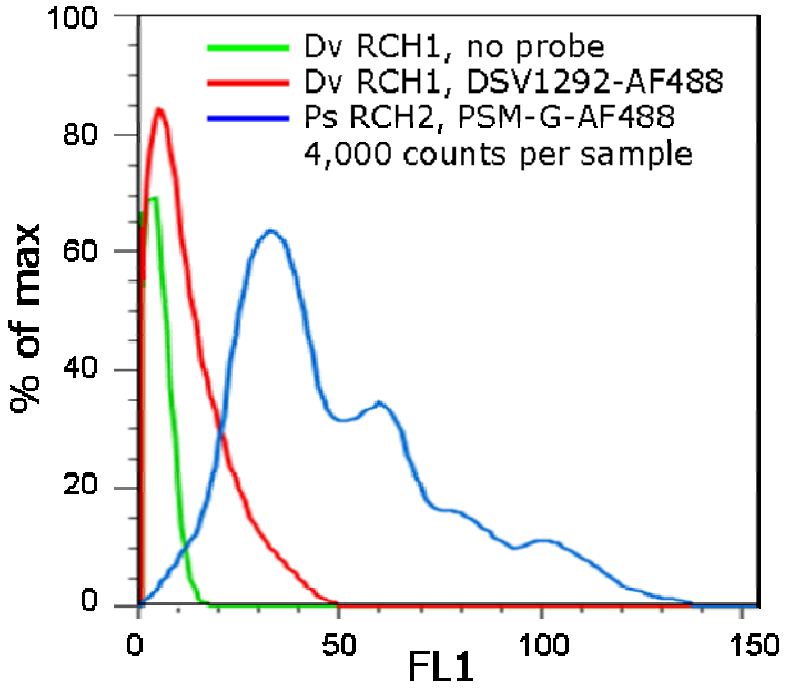
Technique based on optical tweezers
Focused IR laser light at high power

Perroud, Kaiser, Sy, Lane, Branda C, Singh, Patel; *Anal Chem* (2008)

Chip Flow Cytometry

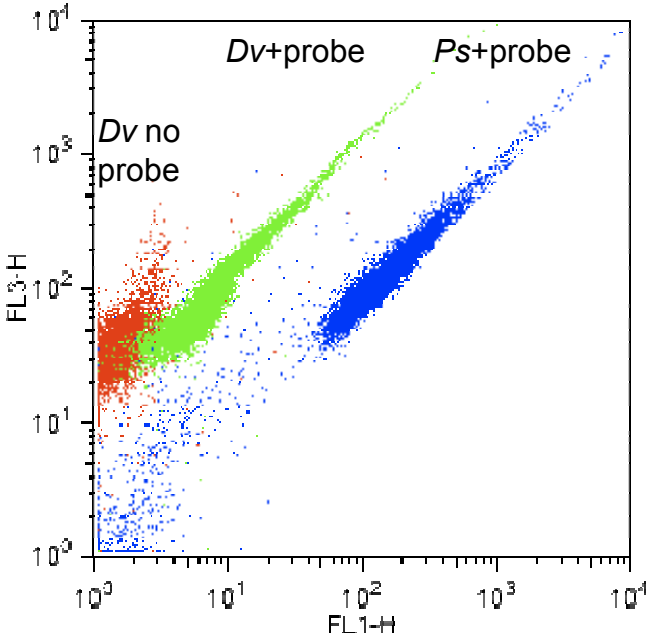
- We have built a microscale flow cytometer for analyzing on-chip cell labeling experiments.
- The chip cytometer demonstrates sufficient sensitivity to distinguish FISH-labeled environmental isolates Dv RCH1 and Pseudomonas RCH2 from unlabeled cells (RCH1 has weak signal, RCH2 is much stronger).
- The chip cytometer has sorting capabilities which have previously been demonstrated for bacteria (*E. coli*).
- Chip cytometer has throughput scaled properly for on-chip FISH hybridization.





Chip results are comparable to BD FACScan

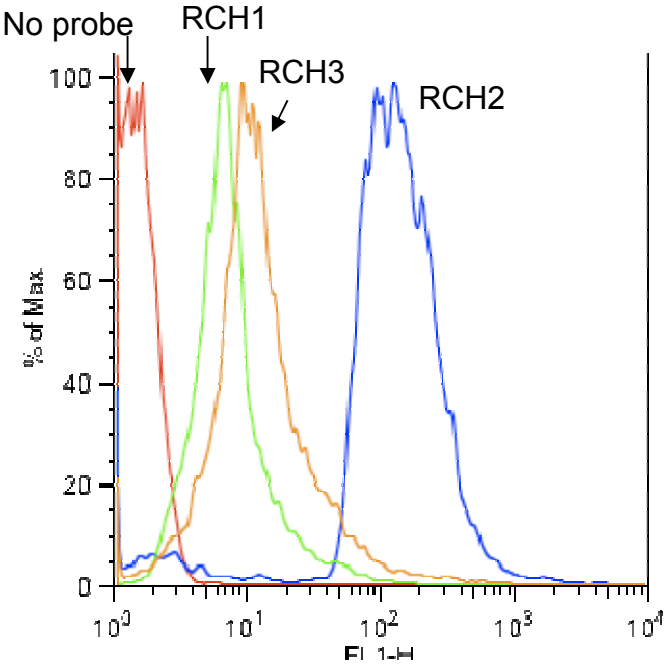
FISH-hybridized *Dv*, *Pseudomonas,* and *Geobacter* (RCH1, RCH2, and RCH3) cells are readily distinguished from unhybridized cells on a conventional BD FACSscan, with similar results to the on-chip cytometry. Sorting based on FISH is also possible with a FACSAria cytometer.



RCH1 + 7-AAD

RCH1, 7-AAD, DSV1292-AF488

RCH2, 7-AAD, PSMG-AF488



RCH1 + 7-AAD, no probe

RCH1 + 7-AAD + DSV1292-AF488

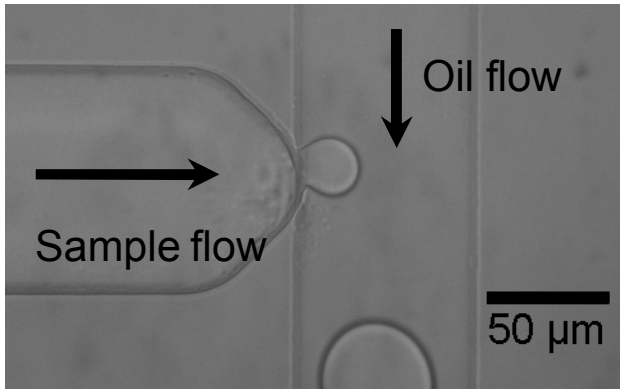
RCH2 + 7-AAD + PSMG-AF488

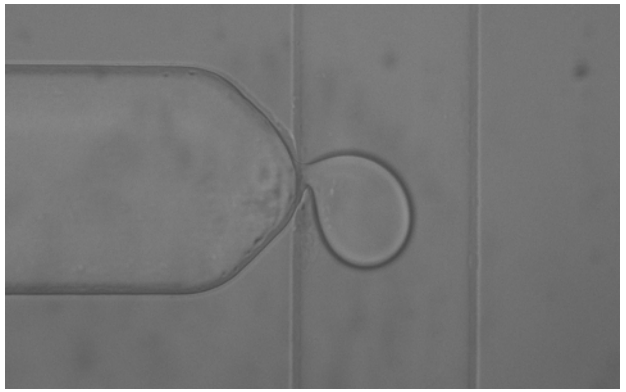
RCH3 + 7-AAD + GEO564-AF488

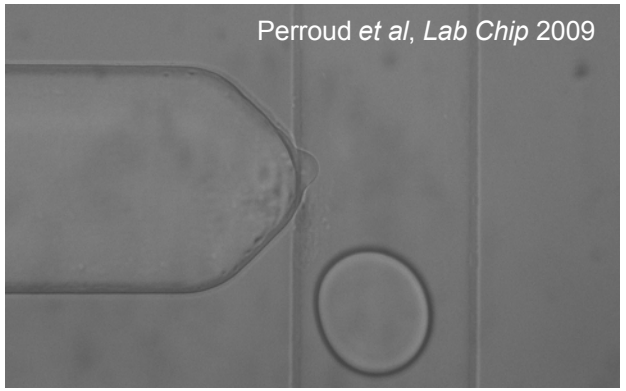
488 nm laser excitation; FL1 = 530/30 nm; FL3 > 670 nm; 10,000 counts per sample
Fixed cultured cells hybridized with FISH probe and 7-Amino-actinomycin D (7-AAD, a far-red DNA stain)

Microdroplets for single-cell compartmentalization

- We can use a microfluidic channel to segment fluids into a series of highly uniform microdroplets with 10-1000 pL volume
- Similar to “emulsion PCR” techniques, except the droplets are generally larger, and much more uniform in size.
- Droplets can be generated at rates of ~10-1000/second
  - many fewer compartments than emulsion PCR
  - many more compartments generated in a few minutes than a microtiter plate
- In glass channels – utilize “radial micropore” etching technique to create droplet orifices that are smaller than the channel.
- Opposite flow regime from 2-phase extraction
  - Small capillary number ($Ca = \mu U / \gamma$)
  - Even with surfactants, γ is relatively large
  - Interfacial forces are dominant



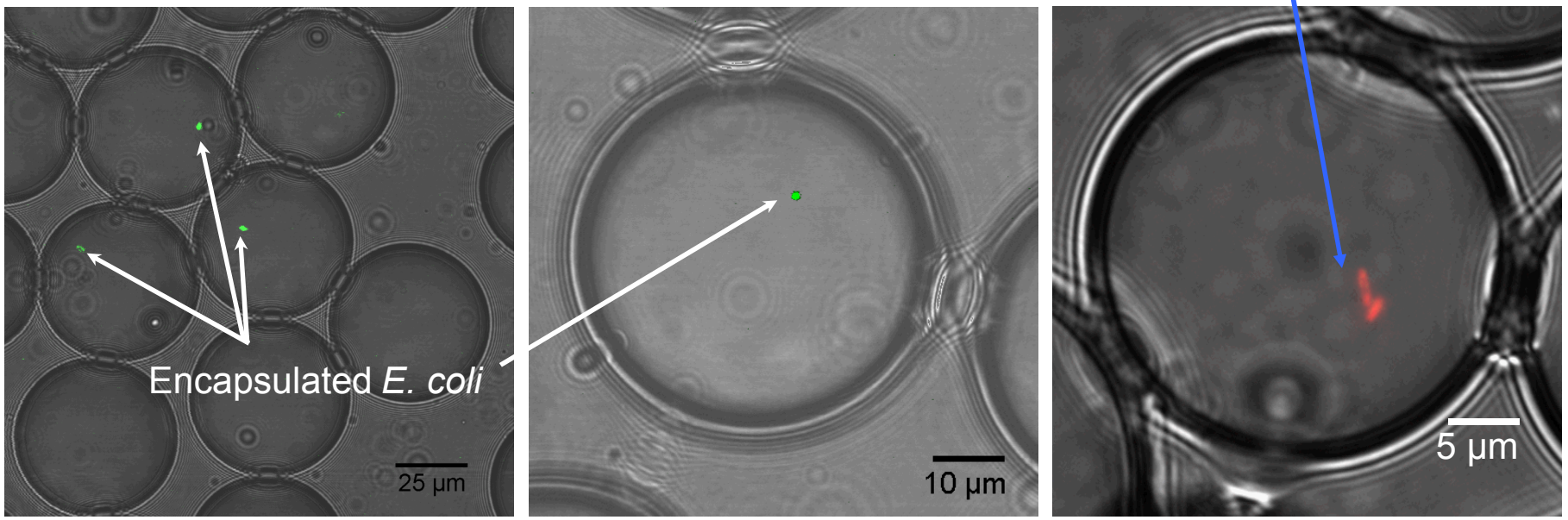




Encapsulating bacteria within droplets

- To ensure single-cell encapsulation, rely upon stochastic loading
  <<1 cell per droplet; most droplets are empty
- Future: on-demand droplet generation
- Goal is to perform a whole-genome amplification in the droplet
  - Small reaction volume has been shown to improve fidelity of ϕ29 polymerase MDA reaction (picoliter volume has not been tested!)
  - A second stage of MDA at larger scale will be needed to generate enough DNA for sequencing.

E. coli labeled with
16S FISH probe



Expected impact

- Combined tools (FISH, sorting, and nano-scale genome amplification) should improve the efficiency of single-cell sequencing studies, with improved fidelity of sequencing.
- For our sponsor (NIDCR) – could lead to enhanced understanding of role of microbial community in inflammation associated with oral disease, *e.g.* OSCC.
- For microbiology community as a whole – single-cell sequencing is a rapidly expanding research objective for many types of sample – human (mouth, gut, skin, etc), enivronmental (bioremediation, etc), enzyme prospecting, metagenomic surveillance, etc.
- Challenges:
  - Integration of multiple disparate functions in a single device is always a challenge, especially with a small team and small budget.
  - Individual technologies can be made to work independently of one another; non-chip versions of each technology can be used while validating each chip process.