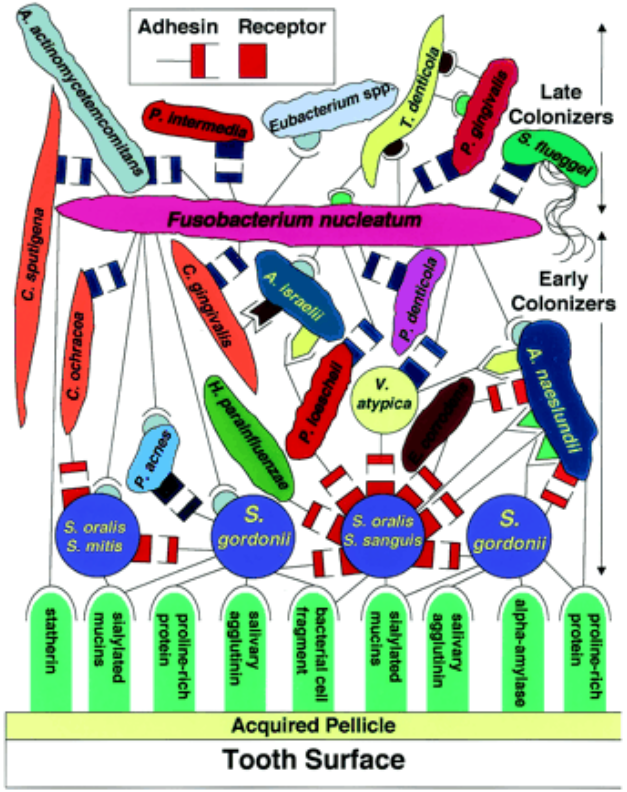
SAND2011-3142C

FISH 'n' Chips: Single-cell phylogenetic analysis of complex microbial communities



Kolenbrander, *et al*., 2002, Microbiology and Molecular Biology Reviews 66:486-505.

Robert J. Meagher, **Peng Liu, Yooli K. Light, Suzan Yilmaz, Anup Singh**
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New York University College of Dentistry

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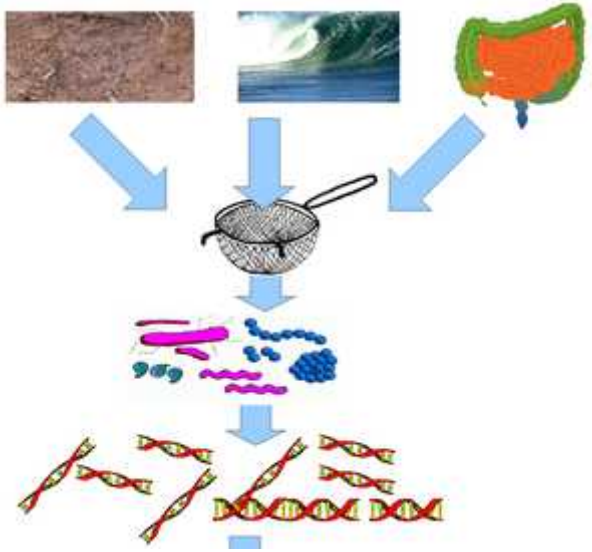
Everything I know about microbiology, in one slide or less

- Bacteria (and archaea) do more than you might think, in the human body and in the environment.
- "Classical" microbiology techniques miss a lot
  - Biased toward things that grow well under defined culture conditions
  - Corollary: a lot of things don't grow well under defined culture conditions
  - Corollary #2: bacteria don't grow in pure culture in nature.
- DNA sequencing reveals a lot that culture-based methods miss, and comes in three flavors:
  - Phylogenetic profiling (e.g. 16S rRNA sequencing)
  - Shotgun metagenomics
  - Whole genome sequencing
- But sequencing and assembly a whole bacterial genome generally requires many cells worth of genomic DNA, which generally requires pure cultures of clonal populations (see corollaries above…)

www.wikipedia.org



Wooley, *PLoS Comput. Biol.* 2010

How can we sequence microbial genomes if we can't culture the microbes?

- Shotgun metagenomics
  - Extract all of the DNA from a population, chop it up into little bits, sequence, sequence, sequence some more
  - Difficult/impossible to assemble genomes or large contigs, except from the simplest communities (*e.g.* <10 species)
  - Useful for cataloging functional genes/metabolic activities in a population, but difficult to link a particular gene back to a particular type of cell

- Single-cell whole genome amplficiation and sequencing
  - Separate a population into individual cells
  - Make many copies of the genome (typically Phi29 polymerase MDA), and sequence
  - Quirky when you get down to individual cells (polymerase artifacts, prone to biases)

How to separate out individual cells?

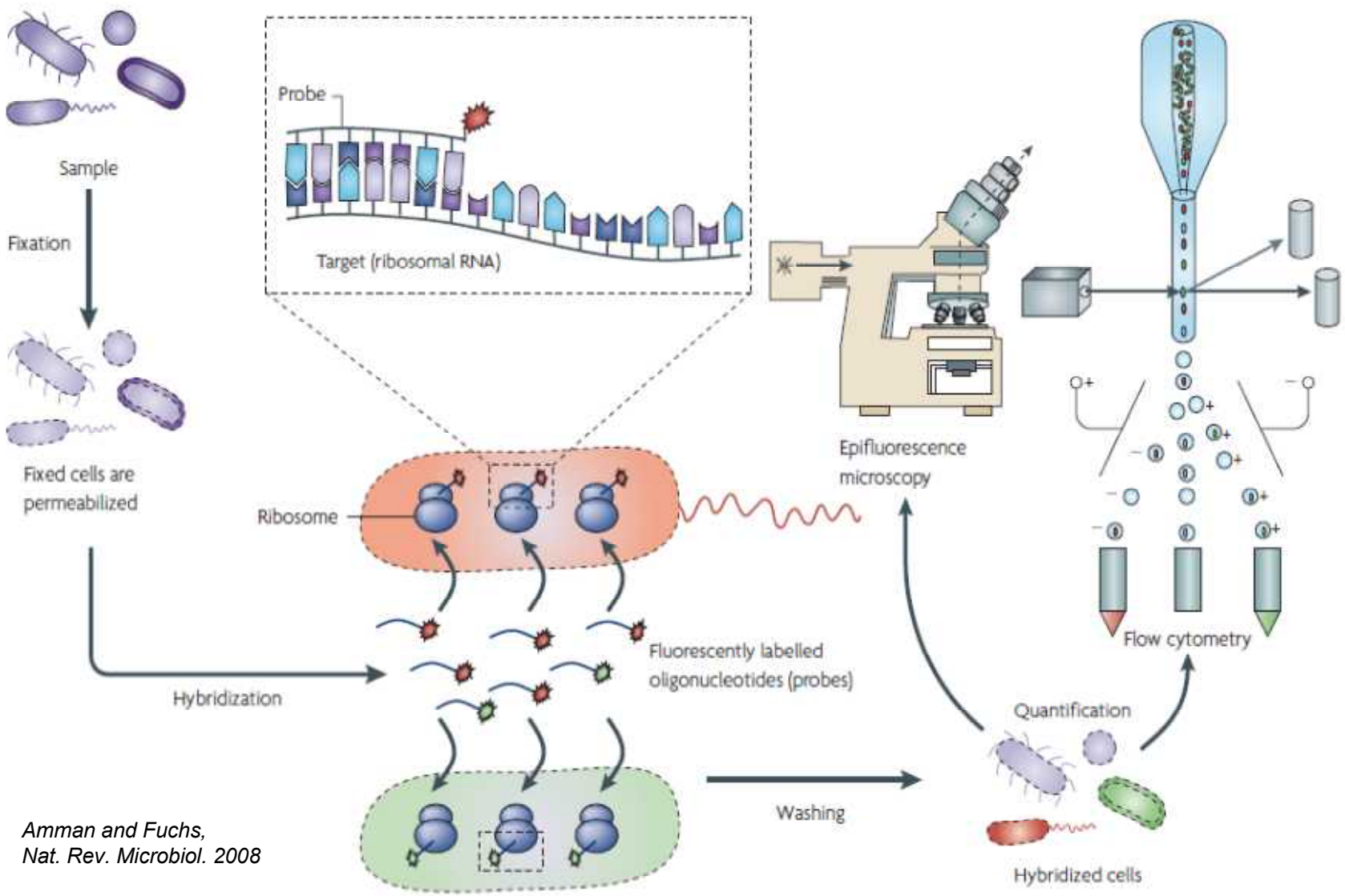
Selecting single cells

- "Blind" sorting
  - Use a flow sorter, or old-fashioned serial dilution, to subdivide a population to $\leq$ 1 cell per well of microtiter plate
  - Perform MDA on everything, limited sequencing (of everything) to see if you have something interesting, and then full sequencing
  - Lots of MDA and sequencing to find interesting bugs

- Targeted sorting
  - Find some way to label cells, and then sort
  - For uncultured cells: antibodies are generally not available. Often the only bit of information we have about uncultured cells is the 16S ribosomal RNA sequence
  - We can label cells using Fluorescence *In Situ* Hybridization (FISH) for 16S rRNA, as a basis for sorting
  - Less sequencing of uninteresting things, although cell fixation for FISH may lead to biases in sequencing coverage.

FISH for bacterial identification

(*FISH = Fluorescence In Situ Hybridization*, aka *Whole Cell Hybridization)*



Amman and Fuchs,
Nat. Rev. Microbiol. 2008

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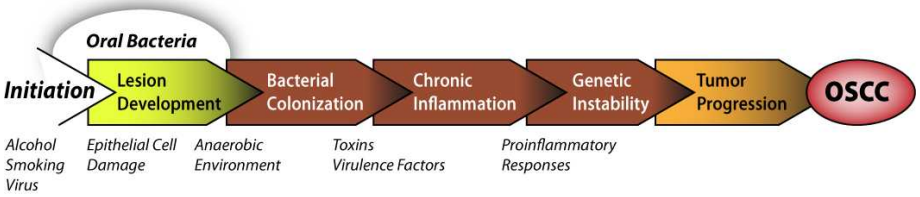
Samples that we care about (and that we have funding to look at)

Oral microbiome (NIH:NIDCR)

The oral cavity has a diverse microbial community (>100 members) and has important, sometimes subtle interactions with human health. We are interested in correlations between bacterial colonization, inflammation, and onset of oral disease (*e.g.* OSCC)

Many of the bacteria of interest are rare and uncultivable but may be correlated with onset of disease.



Courtesy Deepak Saxena, NYU

Hanford site 100H consortia (DOE)

Site was part of the US nuclear weapons complex through ~1970s.

Environmental concerns include contamination of groundwater with Chromium VI (toxic)

Microbial consortium in groundwater has been of interest for possible use for bioremediation, *e.g.* reduction of soluble Cr (VI) to less soluble forms.

>100 species present, but those of greatest interest include *Desulfovibrio*, *Pseudomonas*, *Geobacter*, and *Methanococcus* spp.

To date most studies are done using microarrays (PhyloChip, GeoChip) which lack single-cell resolution.

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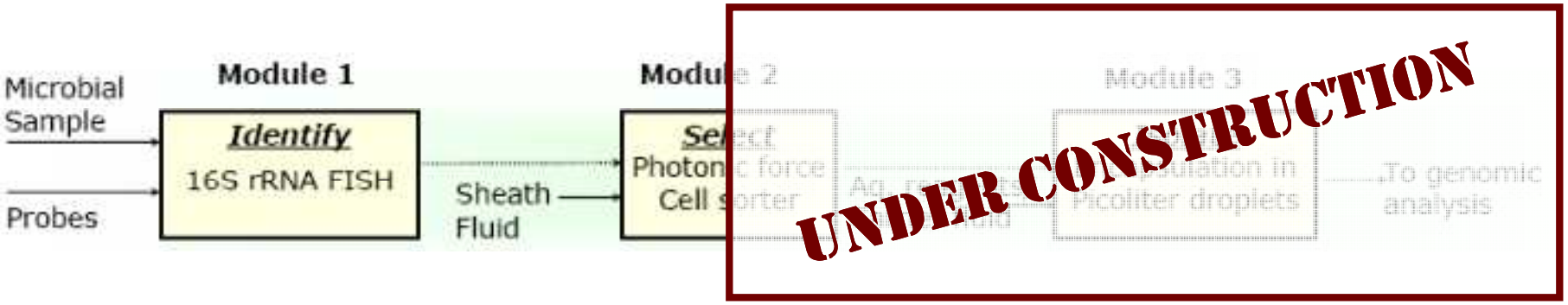
The story so far

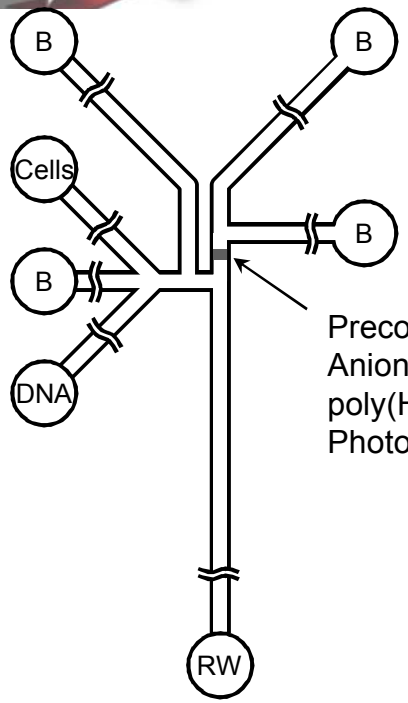
- We would *like* to be able to take small microbial samples, label them with FISH, and sort out single “interesting” cells for WGA/sequencing
- FISH in test tubes with flow cytometry is great for large samples, but you lose a lot of cells doing all the centrifugation/wash steps
- So we are working on a chip to do the FISH labeling, flow cytometry, sorting, and amplification for small/precious samples with not so many cells.
- So far, we’ve got the FISH and cytometry working…



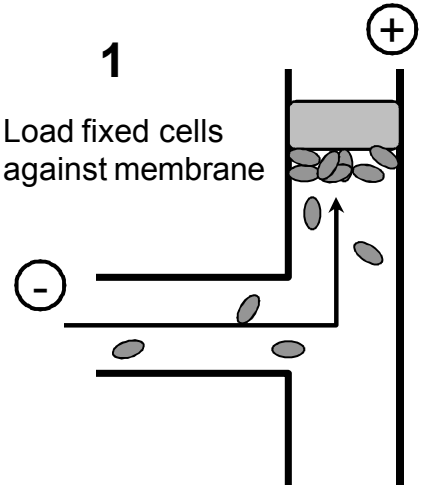
FISH ‘n’ Chips

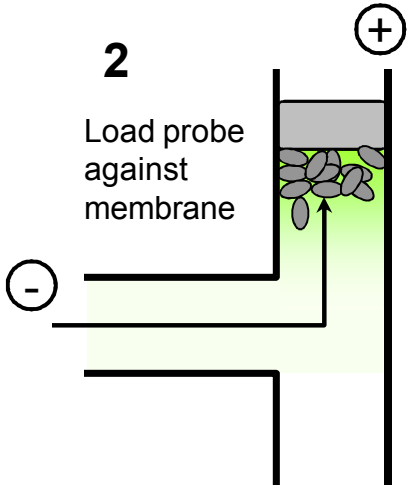
- To recap: in FISH we are hybridizing dye-labeled oligonucleotide probes to the 16S rRNA within intact (but, alas, dead) bacterial cells.
- FISH, the old-fashioned-way, is either
  - Performed with cells fixed on a slide for imaging
    - no further genetic testing possible
    - half your cells are photobleached while you try focus and adjust your camera
  - Performed in suspension or on a filter, for subsequent flow cytometry / sorting
    - many cells are lost in the process, which is Bad if you don’t have many to start with.
- So we have created a chip where we can perform FISH on small samples (<1000 cells).
  - Cells are kept in suspension
  - Good recovery between incubation and wash steps
  - Flow cytometry is performed on the same device

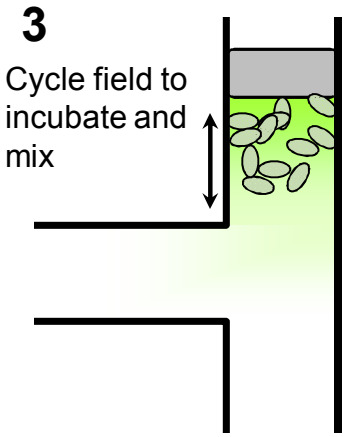
My First try at FISH on a Chip

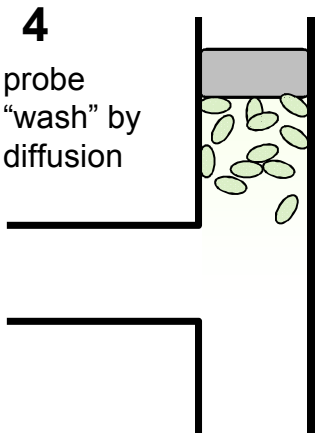


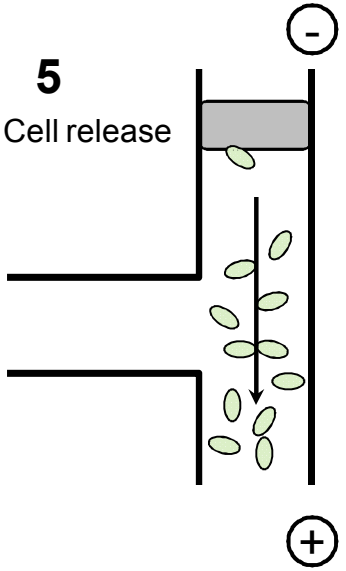
Preconcentration membrane
Anionic, poly(Am-co-AA) or poly(HEMA-co-AA)
Photopatterned *in situ*



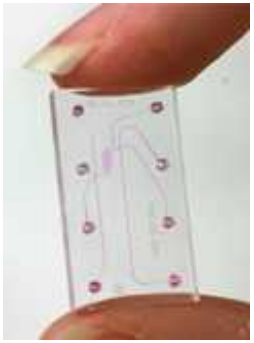


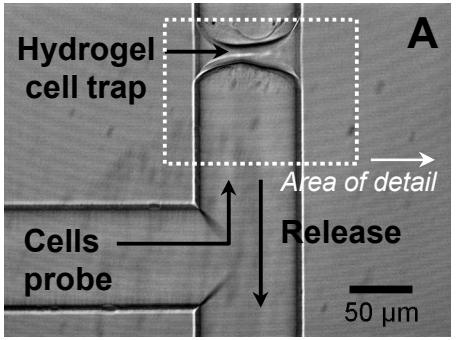




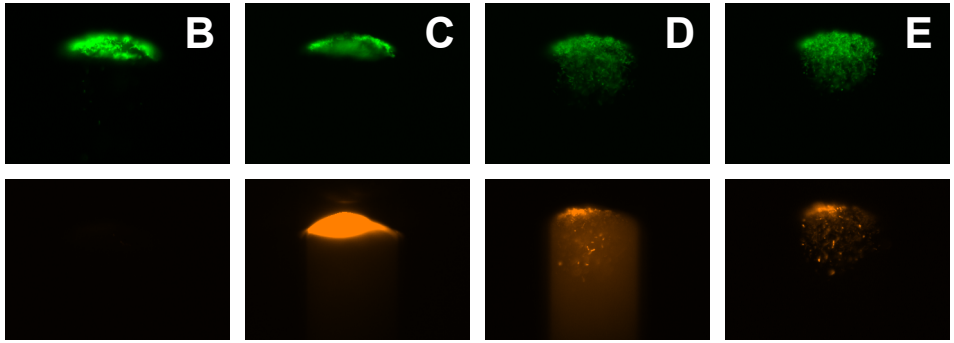


Proof of Concept with Cultured Microbes





Proof of concept chip



GFP Cells loaded

Incubation with Cy3 probe

Wash by diffusion

Cy3 probe labeled cells

FISH with cultured cells of human significance

	E. coli (Gram -)		*L. acidophilus* (Gram +)		*S. mutans* (Gram +)	
probe	Off chip	On chip	Off chip	On chip	Off chip	On chip
NON338	-	Weak	-	-	-	-
Eco681	+	+	-		-	
LAB158	+	+	+	+	-	
Mut590	-		-		+	+

Not great results, but FISH on chip worked similar to off-chip.

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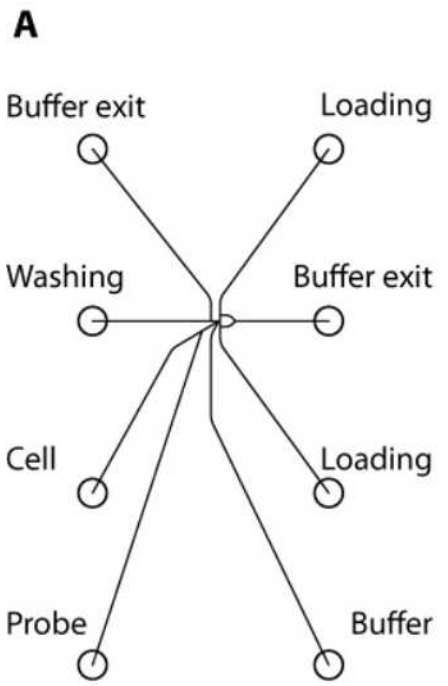
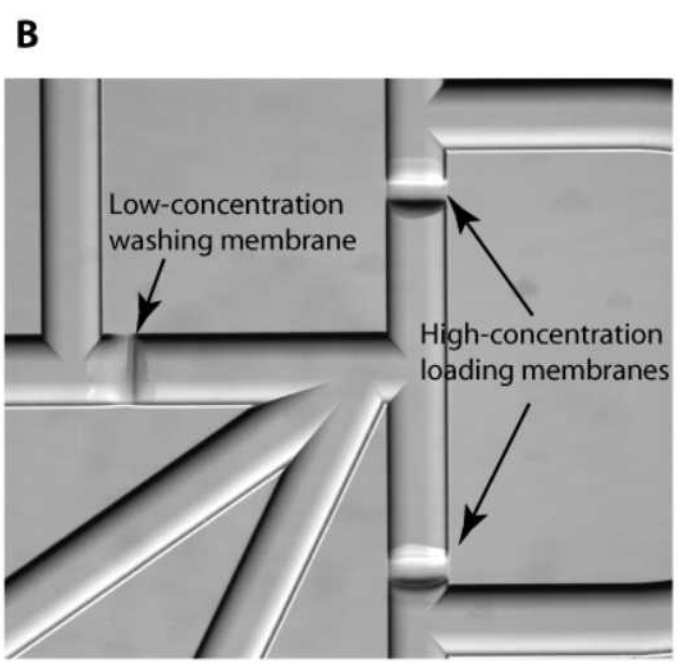
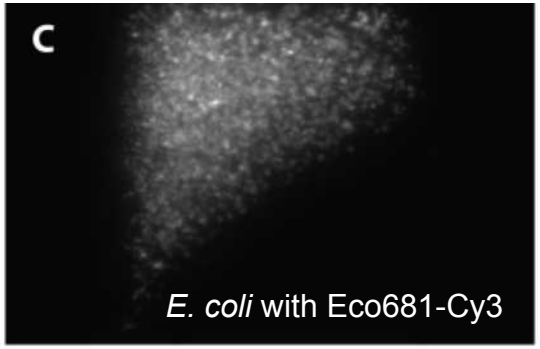
Redesigned chip: iteration 1

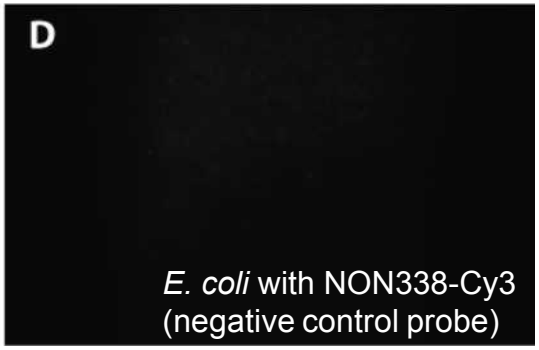
- New design added space for three membranes: two high concentration “incubation” membranes, and a low concentration “washing” membrane (permeable to probe)





Meagher *et al*, *MicroTAS Proceedings* 2010





- Overall field is low (< 20 V/cm)
- Cells tend to clump at high salt concentration
  - Incubation at 400 mM NaCl; washing at 50 mM NaCl (higher stringency)
- Cell migration (*i.e.* zeta potential) depends on pH, salt, cell type, etc.
- Charged membrane isn’t really necessary for blocking probe

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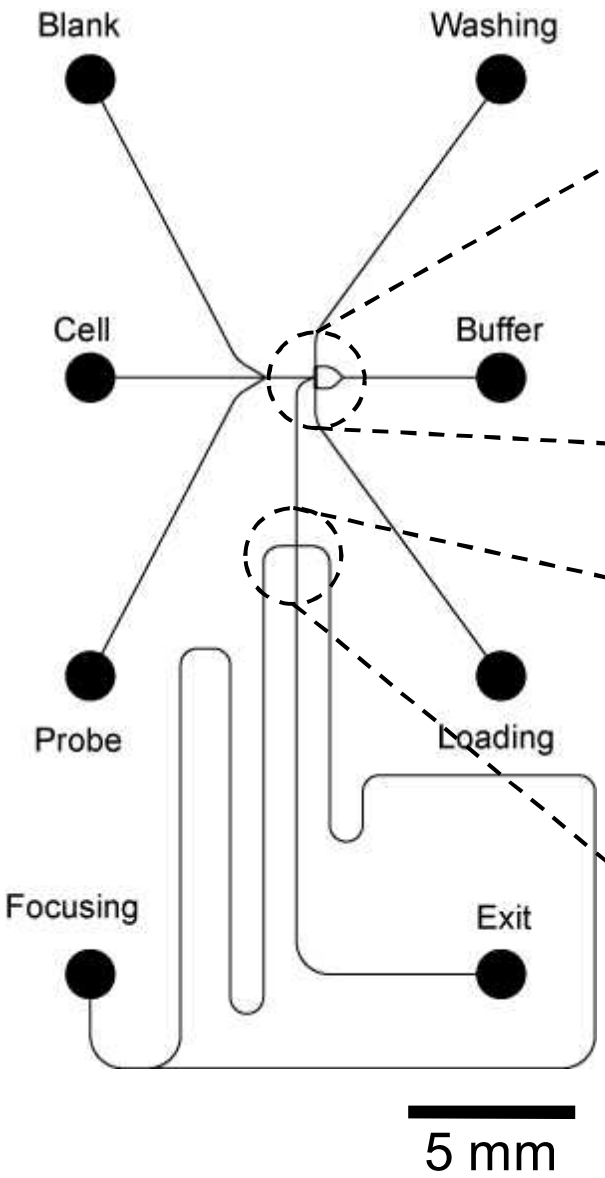
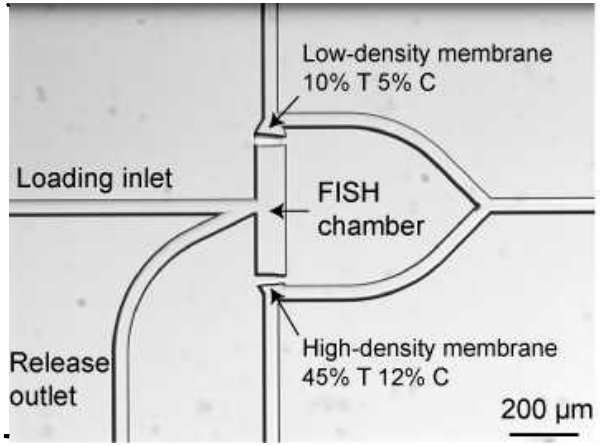
Iteration 2: FISH-Flow Cytometry Chip

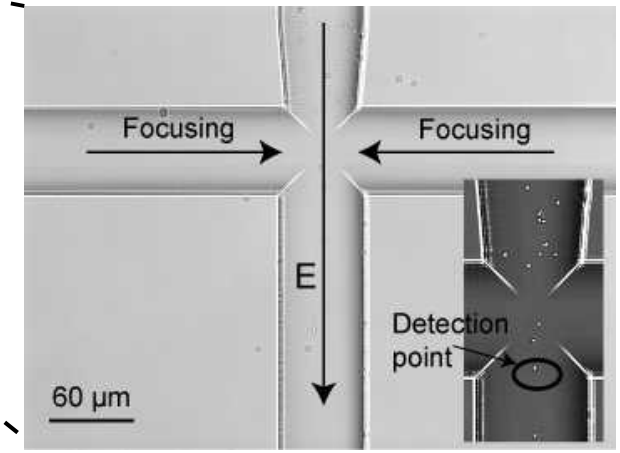


FISH Chamber



1. High-density membrane for concentration and incubation.
2. Low-density membrane for washing.

Electrokinetic Focusing



1. Speed: ~3-5 cells/s.
2. Laser induced fluorescence and forward scattering detection system.

P. Liu *et al*, in prep

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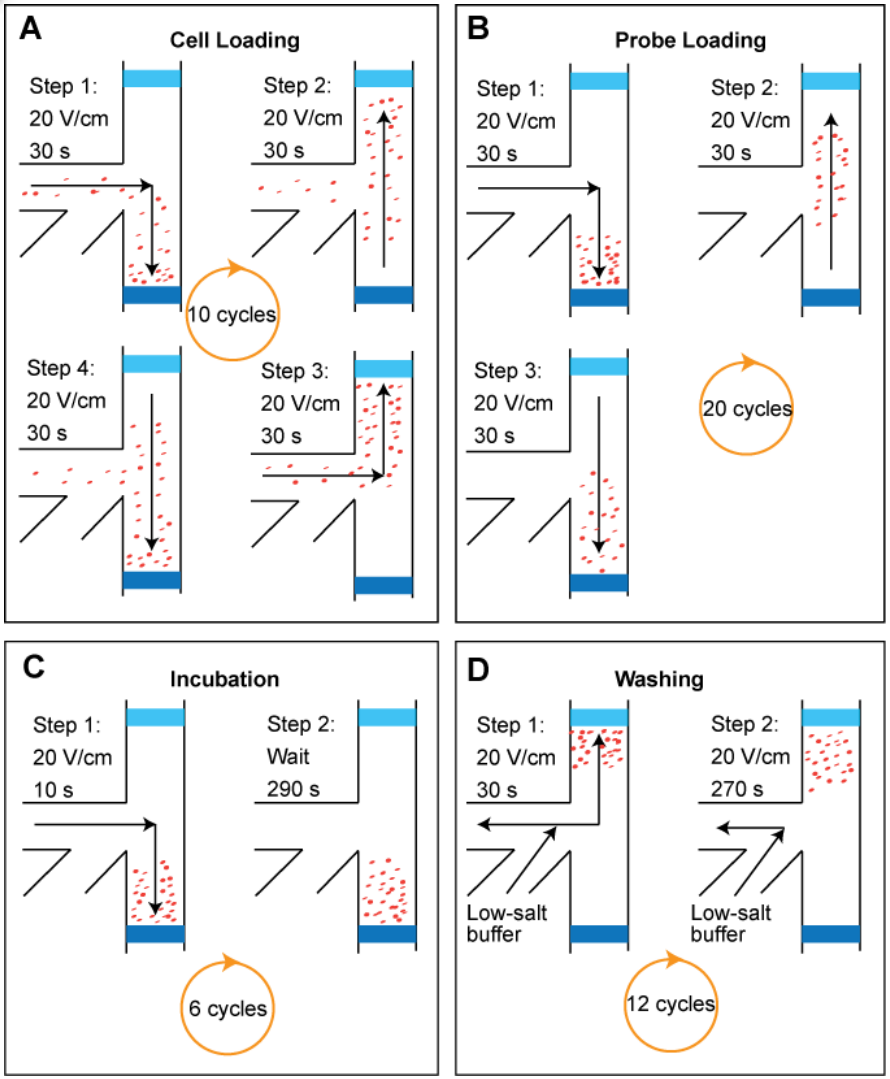
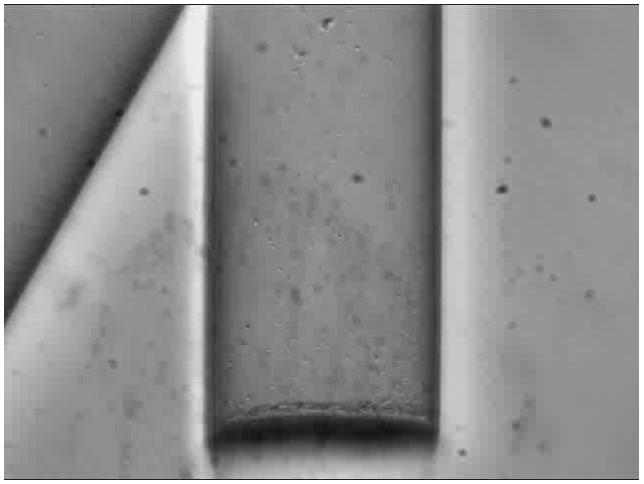
Protocol for FISH and Flow Cytometry

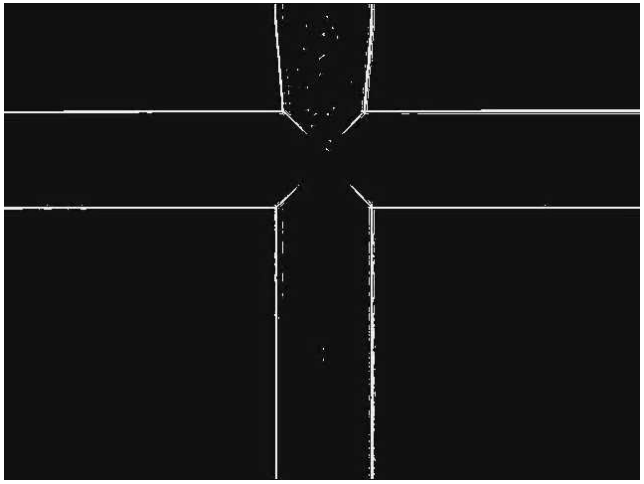


Schematic of the on-chip FISH process.
The total analytical time is 2.5 hr.

Cell loading process



Electrokinetic focusing for flow cytometry



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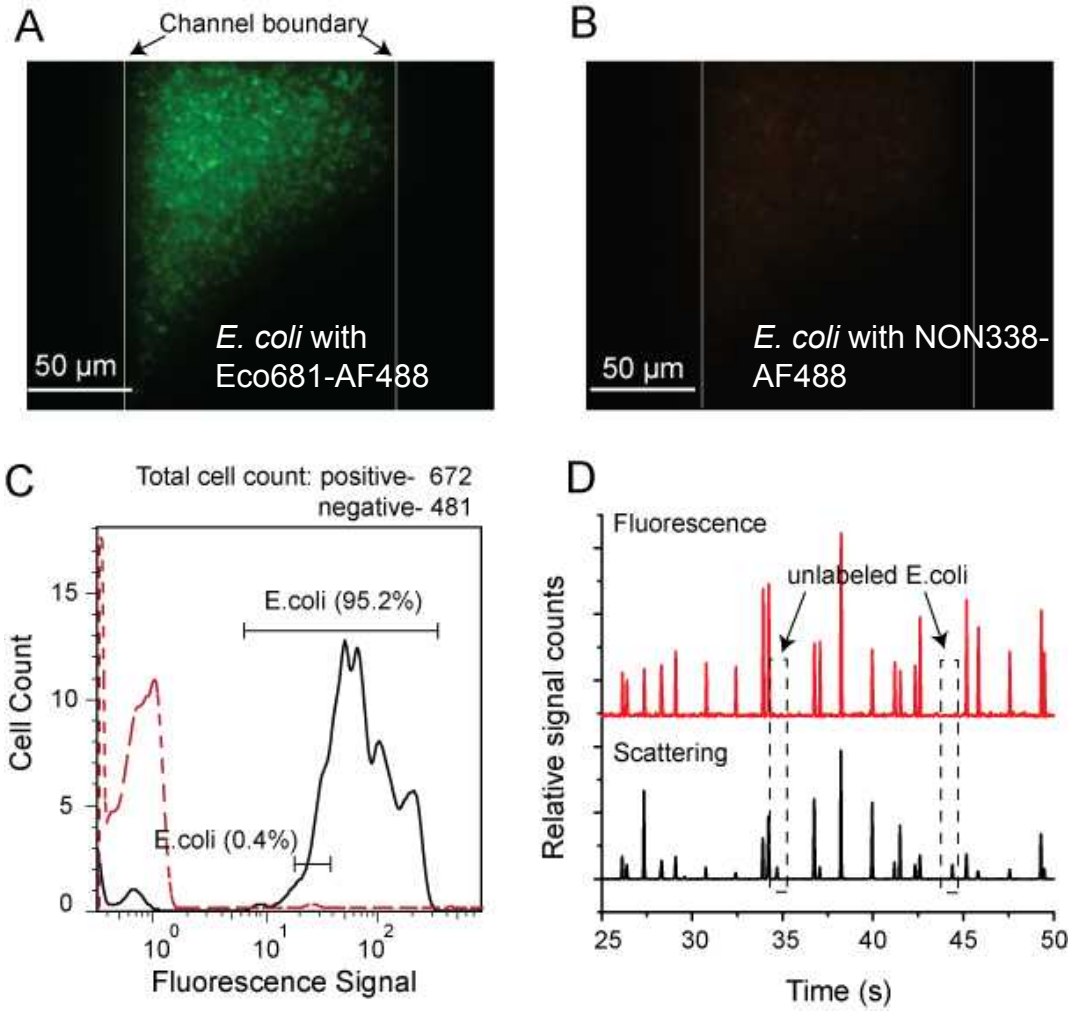
Proof of concept and optimization with *E. coli*



On-chip flow cytometry works pretty well (for now, just a blue laser, forward scatter and green fluorescence are working)

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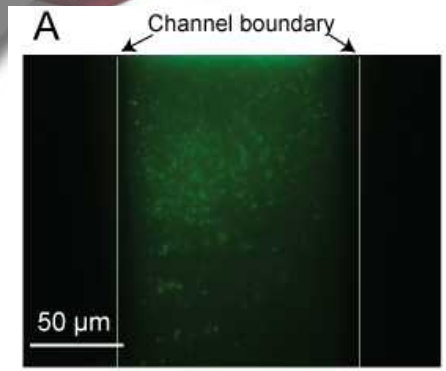
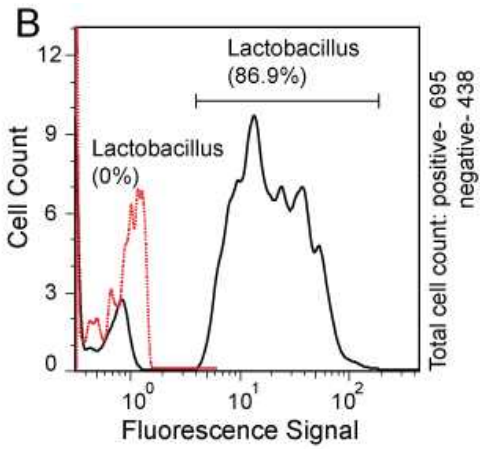
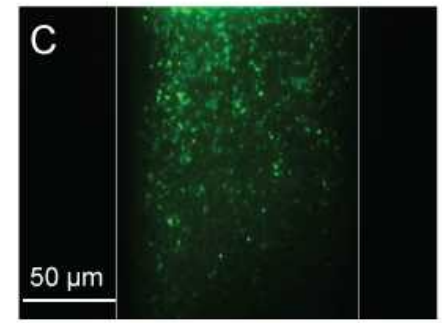
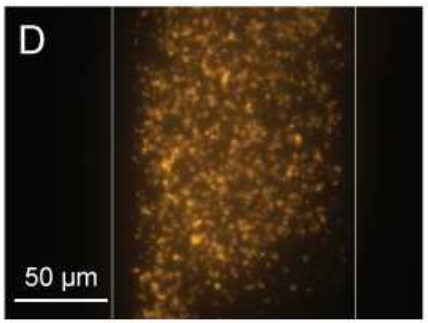
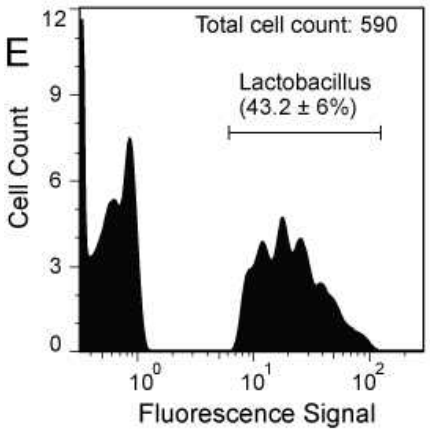
And it works with Gram-positives too…

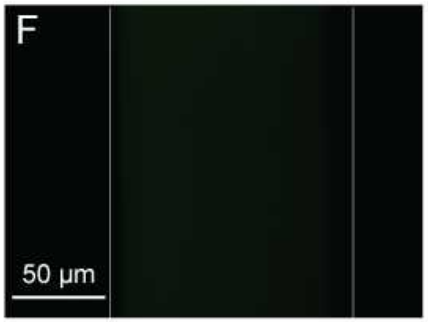












Lactobacillus acidophilus was treated with lysozyme before FISH.

A. On-chip FISH of *Lactobacillus* with Alexa488-labelded Lab158 probe.
B. On-chip flow cytometry results. (Black line: positive with EUB338. Red line: negative with NON338 probes
C. On-chip FISH of the mixture of *E.coli* and *Lactobacillus* (50:50 mix). *Lactobacillus* labeled with Lab158-alexa488.
D. Same mixture, *E.coli* labeled with Eco681-cy3.
E. On-chip flow cytometry results of *Lactobacillus* in the same mixture sample.
F. Negative control of *E.coli* with Lab158 probe.

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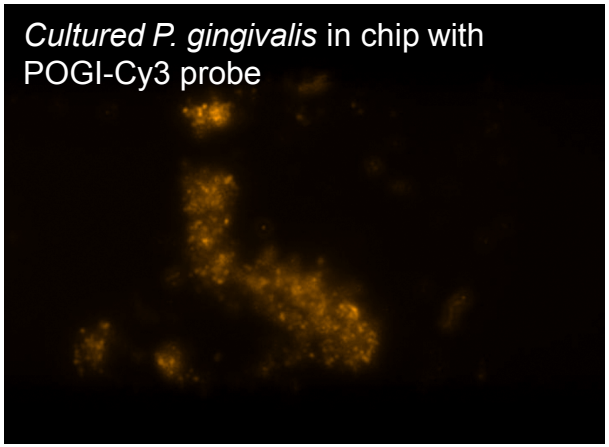
Oral bacteria

FISH on a chip compares well to FISH off a chip for the following cultured species of oral significance:

- *L. acidophilus* (Gram positive, “probiotic”, surrogate for other oral *Lactobacilli)*
- *S. mutans* (Gram positive, causative agent of dental caries)
- *P. gingivalis (*Gram negative, involved in periodontal disease and inflammation)
  - Tend to clump together inside the chip – not ideal for FC

We’re expecting (healthy) saliva samples from Deepak any day now…

- Saliva contains representatives of most of the hard and soft sites within the mouth (teeth, gums, palate, *etc*.)
- Samples will be fixed and washed outside of the chip
- To be determined if cells from saliva will clump, stick to membrane, etc.



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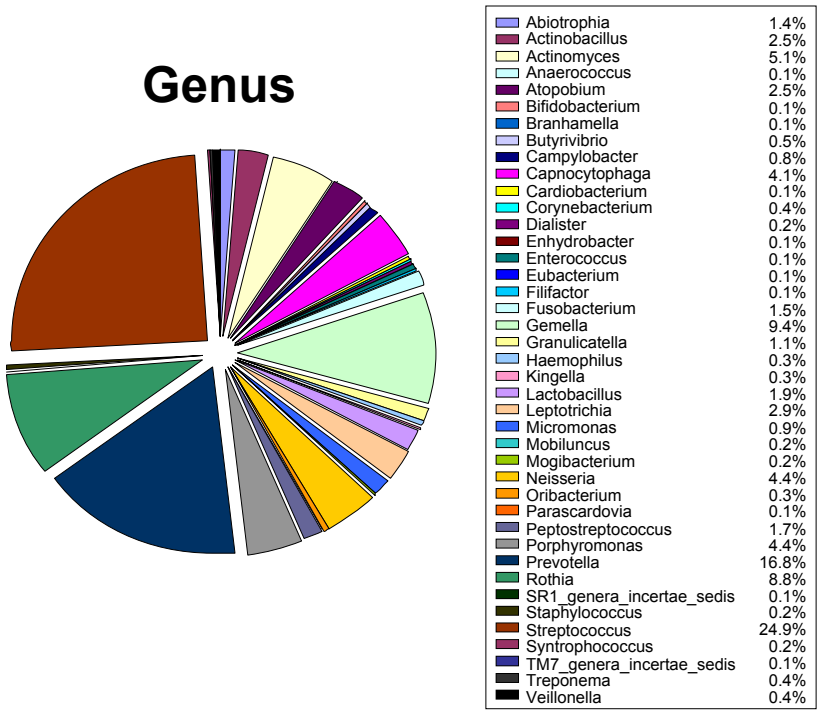
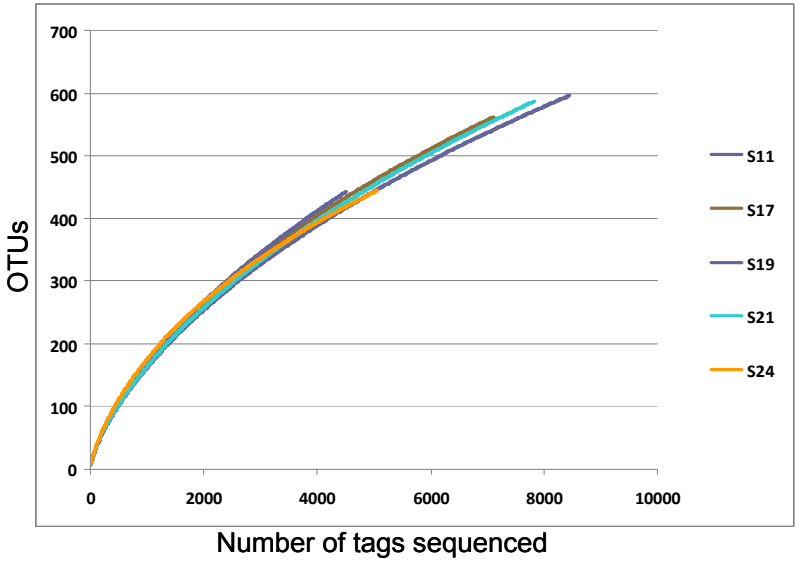
What can we expect to find in the mouth?

454 sequencing of V4-V5 of 16S rRNA from 2 OSCC and 6 healthy patients





Courtesy of Deepak Saxena, NYU

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Selected bacterial phylotypes identified in either of OSCC subjects and their putative virulence properties

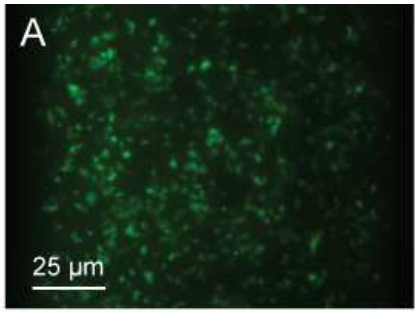
Bacterial Phylotypes	Characteristics
Actinomyces georgiae	oral biofilm
Actinomyces naeslundii	oral biofilm
Bifidobacterium breve	intestinal inflammation
Capnocytophaga spp	periodontal diseases, septicaemia
Clostridium butyricum	intestinal toxemia botulism
Prevotella melaninogenica	oral cancer
Tissierella praeacuta	
Fusobacterium necrophorum	septicemia, tonsillitis
Gemella haemolysans	Meningitis, renal failure
Gemella sanguinis	infective endocarditis
Parvimonas	apical abscesses
Peptostreptococcus micros	chronic inflammatory periodontitis, rheumatoid arthritis, OSCC
Porphyromonas gingivalis	extraoral and Some odontogenic Infections
Prevotella intermedia/nigrescens	bacteremia
Rothia mucilaginosa	infective endocarditis
Staphylococcus saccharolyticus	human inflammatory bowel diseases, others isolated from soil, lake, gut microbes deep-Sea
uncultured bacterium; EF705934, EF700494, AF468245, AY209384, AB355083	bronchoalveolar lavage
uncultured epsilon proteobacterium; AY280397	bacteremia
uncultured Streptococcus sp AY806067	
uncultured unknown (total of 43)	
Veillonella spp	

Courtesy of
Deepak Saxena, NYU

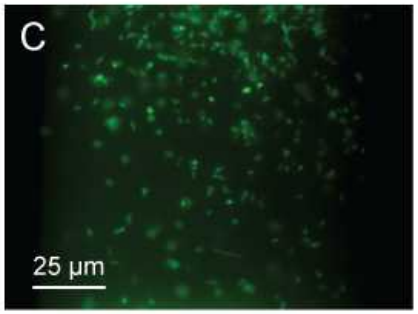
FISH on Chip with Hanford isolates

- FISH on a Chip tested with two cultured isolates
  - "RCH1" = *Desulfovibrio vulgaris* strain RCH1
  - "RCH2" > 99% sequence similarity to *Pseudomonas stutzeri*
  - Both are of interest for metal reduction and associated metabolic pathways (nitrate reduction, sulfate reduction, *etc*.)

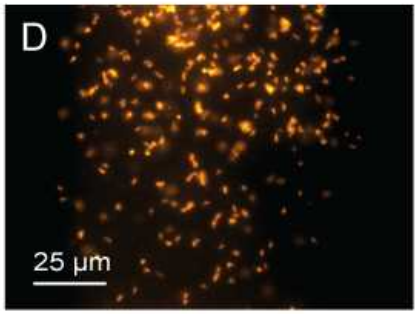
RCH1 & RCH2 (50:50 mix)
With EUB338-AF488 probe
(all bacteria / positive ctrl)



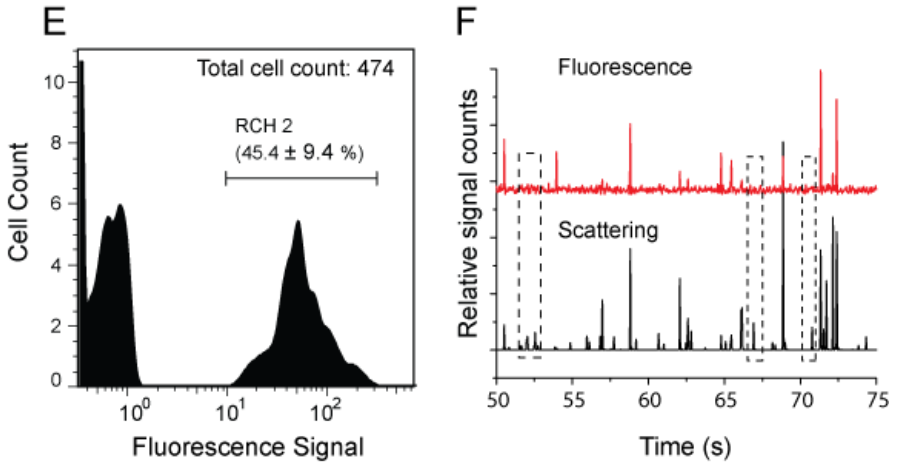
RCH1 & RCH2 (50:50 mix)
With NON338-AF488 probe
(nonsense / negative ctrl)



RCH1 & RCH2 (50:50 mix)
With PSMG-AF488 probe
(Pseudomonas spp.*)*



Same expt as (C)
With DV1292-Cy3 probe
(Desulfovibrio spp.*)*



Flow cytometry distinguishes labeled population AF488 channel / *Pseudomonas* spp. (similar to initial proportion of RCH2 in the mix).

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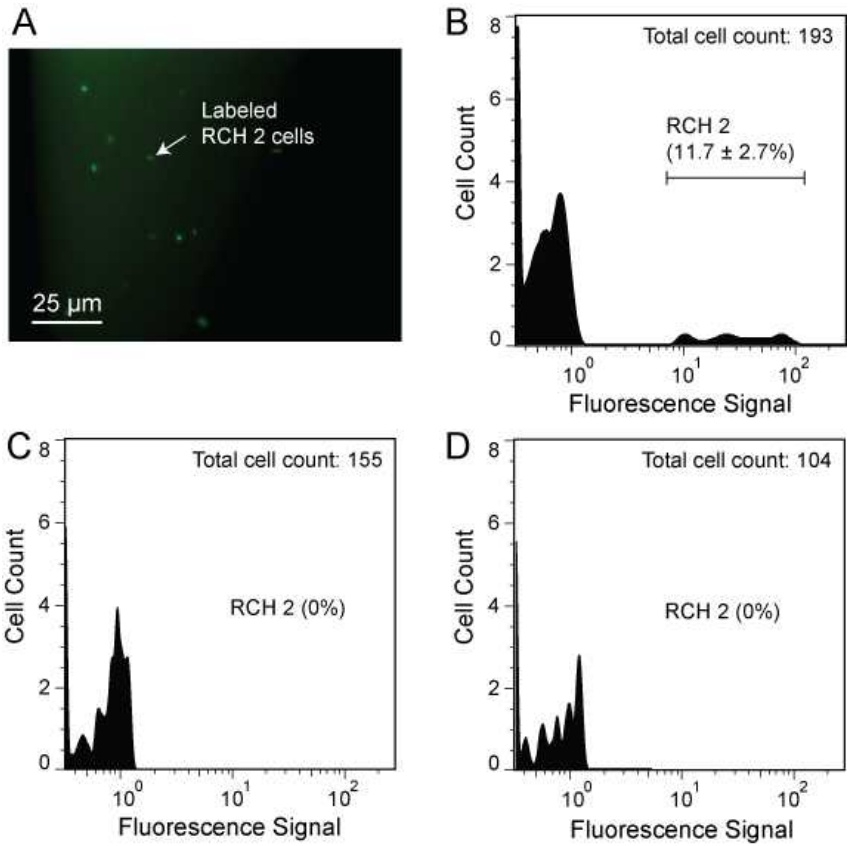
FISH on chip with a “real” sample

- Hanford water from three sampling dates in fall 2009
  - Cells preserved at -20 °C on filter membrane for ~ 1 year
  - Filter incubated and vortexed to release cells
  - Cells loaded on FISH chip for detection of *Pseudomonas* spp. with PSMG probe



Conventional FISH/FC (with BD FACSAria) verifies 10-15% of counts in Sample (B), and <1% of counts in Samples C-D, are labeled with PSMG probe.

FISH is performed on a real environmental sample, with <200 cells.

This particular sample is not so precious, but it verifies that we could run FISH on a very limited sample, with results that agree well with conventional flow cytometry.

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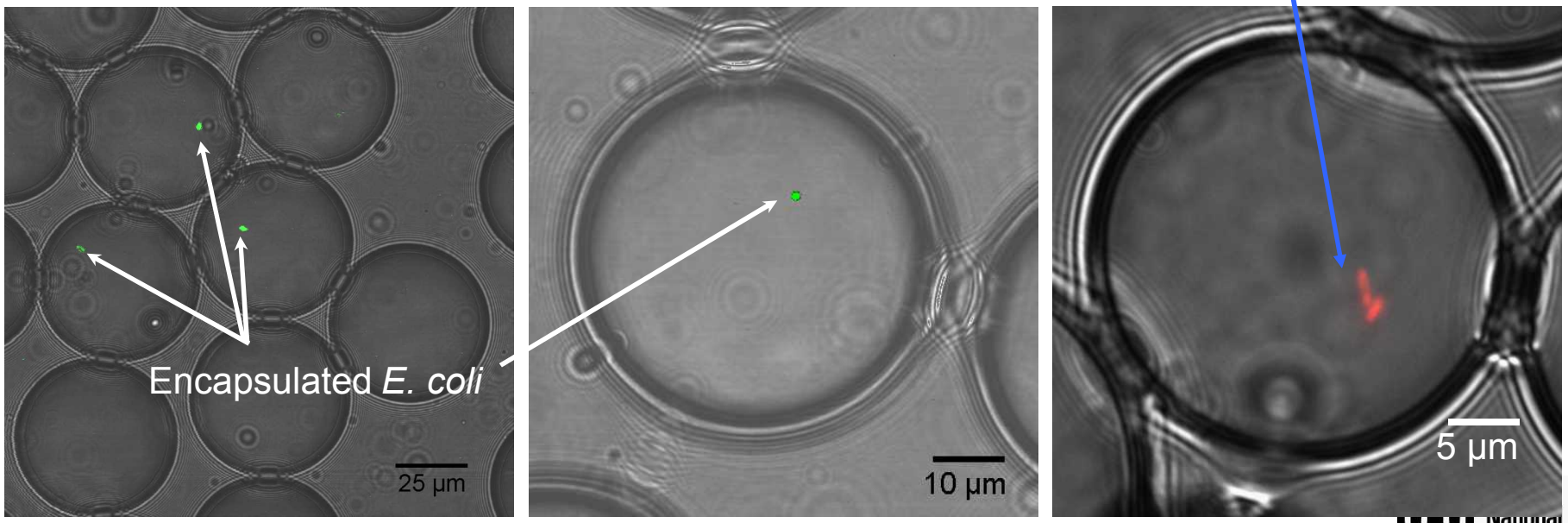
After FISH – then what?

- If we can do FISH-FC, it shouldn't be that hard to sort cells
- We already have a couple of designs of sorter working
- But how to best take advantage of chip scaling?
- Conventional BD FACS – can sort single cells into wells of microtiter plate (microliter volume)
- On chip – can we sort cells into a much smaller volume?

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



“FISH-n-Chips” Bacterial Cell Processor

Value is ability to integrate sorting and single cell encapsulation to get amplified genomic DNA derived from a single *uncultivable* cell

	Module 1 **Hybridization and Incubation**	**Module 2** **Photonic Force Cell Sorting**	**Module 3** **Cell Encapsulation in Picoliter Droplets**
INPUT	• Fixed, permeabilized bacteria • FISH probes for 16S rRNA	• Hybridized target cells • Unlabeled cells • Sheath fluid	• Hybridized target cells • Emulsifying oil stream
STEPS	1. Mix cells with probes and buffer 2. Incubate while mixing 3. Wash away excess probe	1. Hydrodynamic focusing 2. Flow cytometry using hyperspectral fluorescence spectroscopy for decision making 3. Photonic-force cell sorting	1. Microdroplet formation at microfluidic T-junction 2. Rejection of empty droplets 3. Deposition of droplets containing single cells on micropatterned array
OUTPUT	• Hybridized target cells • Unlabeled cells	• Collection channel: hybridized target cells • Waste channel: unlabeled cells	• Individually encapsulated cells • Further genomic testing (PCR, MDA, sequencing) on contents of individual droplets

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FISH n Chips: Conclusions and outlook

- We have developed a microfluidic device for performing FISH labeling and flow cytometry of bacteria from small, precious, or limited environmental samples.
- Most testing thus far has been on cultured representatives, but the true power of FISH is for uncultured cells and for minimally processed environmental samples.
- The protocol requires some fine tuning for specific cell types (including problems with sticking/clumping), and for each probe (adjust salt/temp/formamide for desired stringency).
- New (more interesting?) Hanford samples were recently collected; saliva samples expected soon.
- Next step: integrate flow cytometry with sorting
  - We already have a chip cell sorter based on optical trapping (designed for mammalian cells; may need fine tuning for bacteria)
  - We have a piezo-based sorter under development (works with beads…)
  - So far our FISH process is purely electrokinetic, but our existing sorting techniques are based on hydrodynamic flow
- Down the road: capturing single cells in nanoliter “compartments” for single-cell whole genome amplification and sequencing (c.f. Quake et al).

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CASSS & MSB Organizers

for inviting me!

★ Did most of the hard work in the lab

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