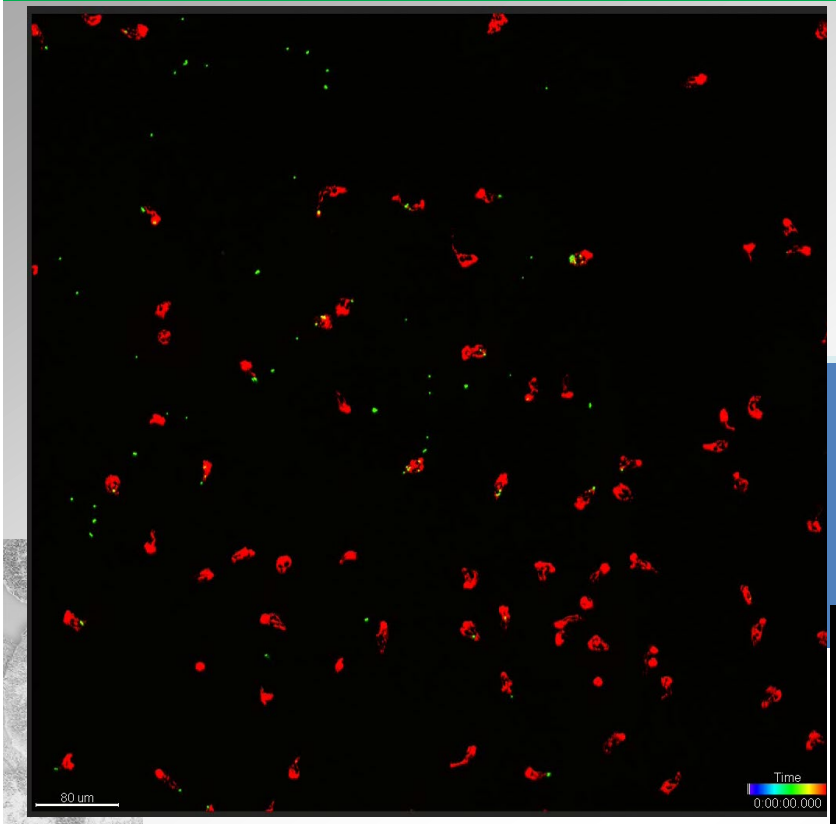
MONTANA STATE UNIVERSITY

Center for Biofilm Engineering

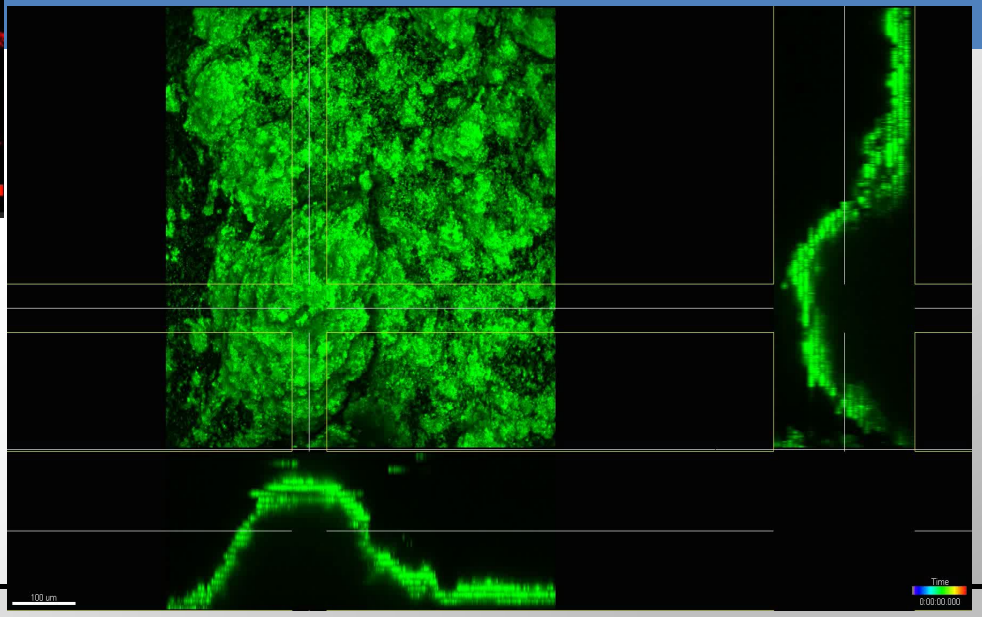


Why do we need a Biofilm Imaging Library?

Current limitations when analyzing confocal 3D images

Al Parker, Biostatistician, CBE

Associate Research Professor, Math and Stats, MSU

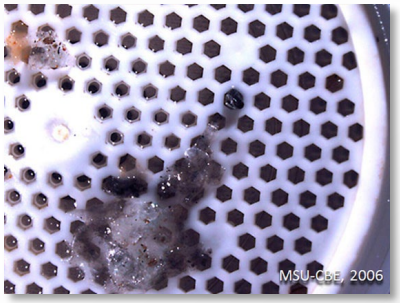


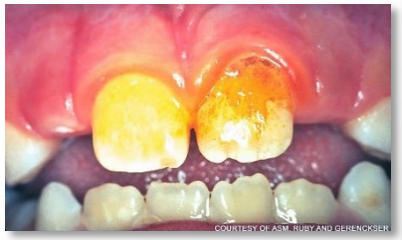
SPMB IEEE

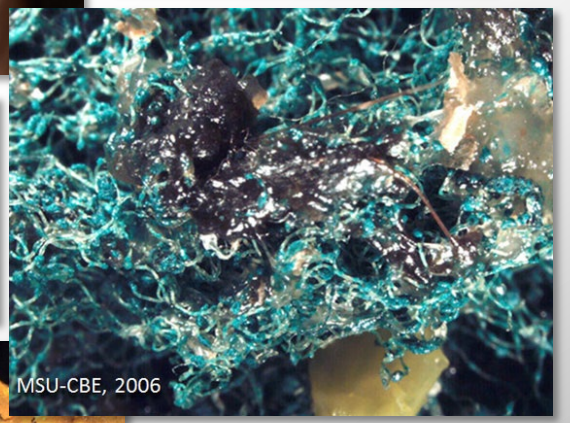
December 6, 2025

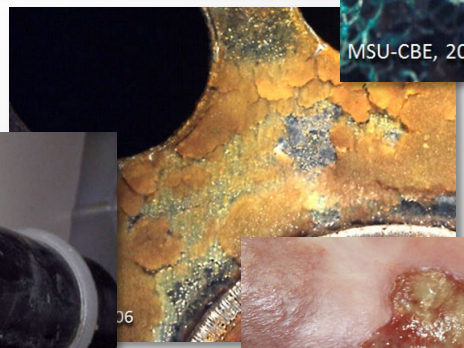
What is a biofilm?

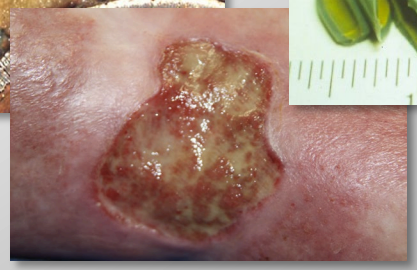
bi·o·film (bī'ō-fĭlm') *noun*:
Microorganisms living in a
self-organized community
attached to surfaces, interfaces,
or each other, embedded in
a matrix of extracellular polymeric
substances of self-origin that
exhibits altered phenotypes/behavior
compared to free-living, planktonic cells.









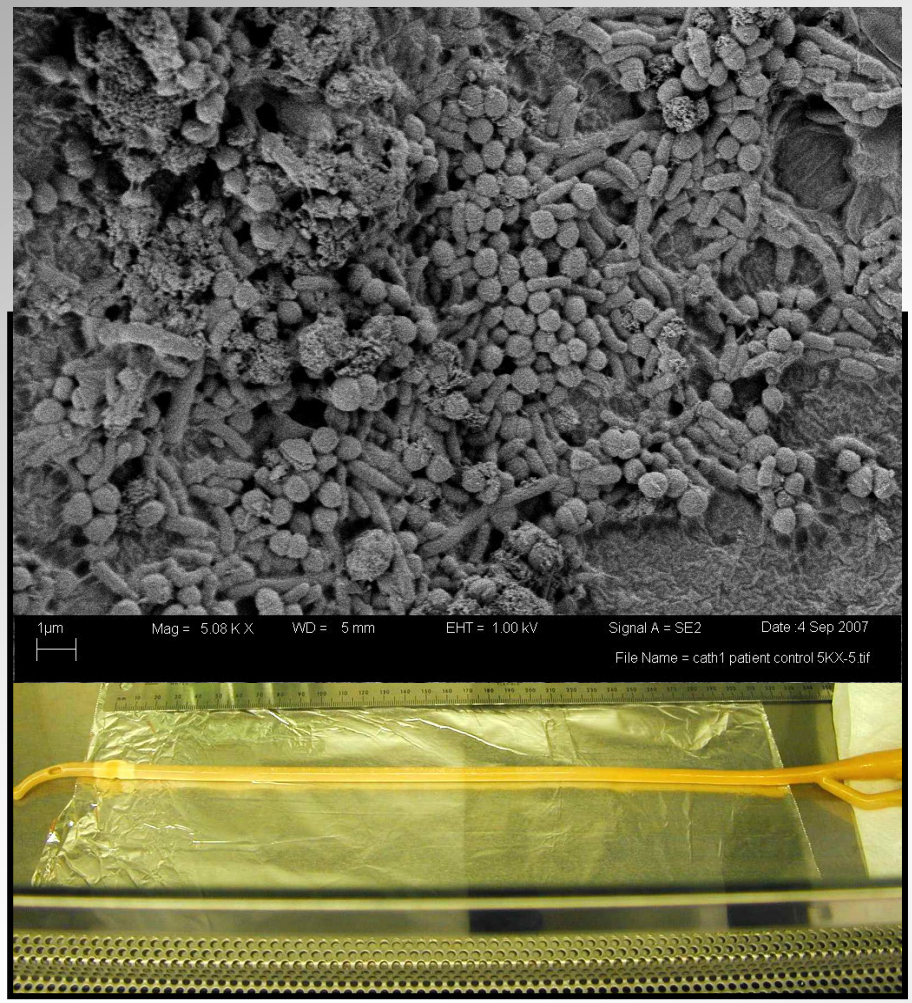


Biofilms can make your beer taste funny

Cleaning draught beer lines

Brewer's Association

Biofilms grow in urinary catheters

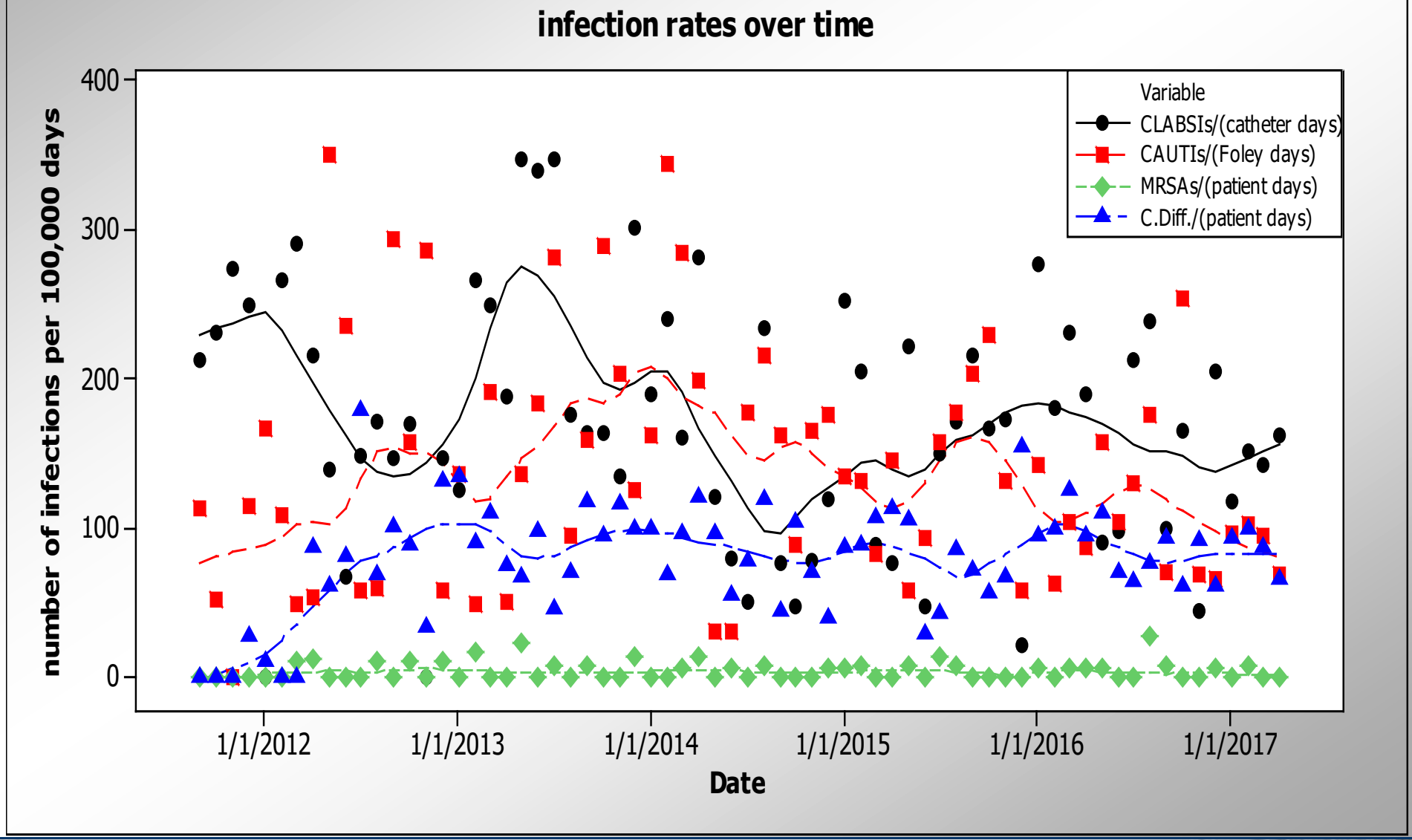


Assessing surface modified urinary catheters

Burroughs Wellcome Fund (Summers at al. in prep)

Biofilm hypothesis: culprit for hospital acquired infections

From 5 hospitals in the US:



Center for Biofilm Engineering

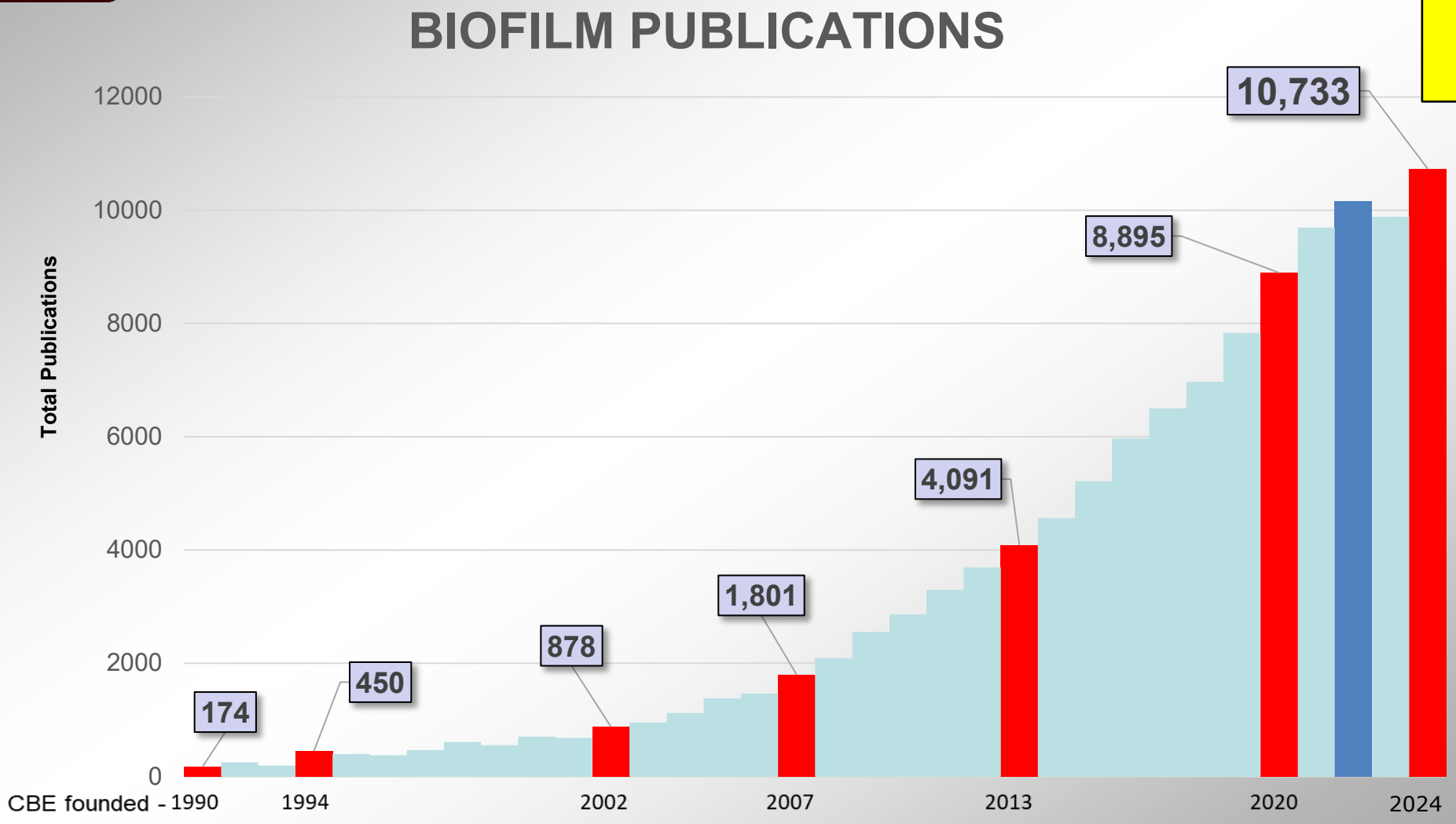
CENTER FOR BIOFILM
CBE
ENGINEERING
35 years

NSF Engineering Research Center Established in 1990

World's oldest, largest, and best-known research center for biofilms

Ignited the Field of Biofilm Research

Pubs have doubled approx. every 6 years



Source: Web of Science

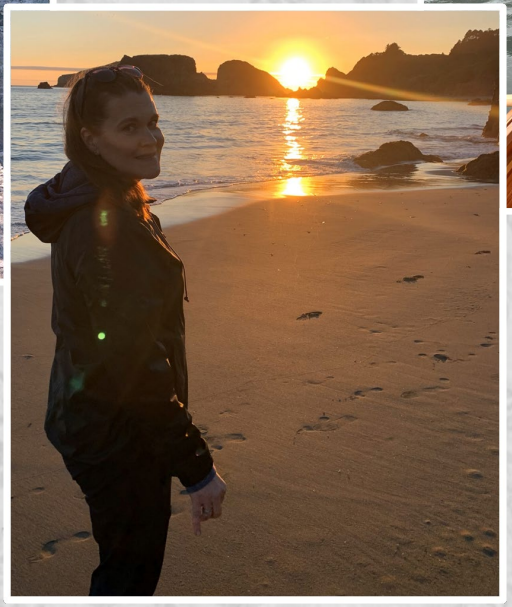
Industry Membership Program

Standardized Biofilm Methods Laboratory

Darla Goeres

Chris Jones

Lindsey Miller



Kelli Buckingham-Meyer

Al Parker

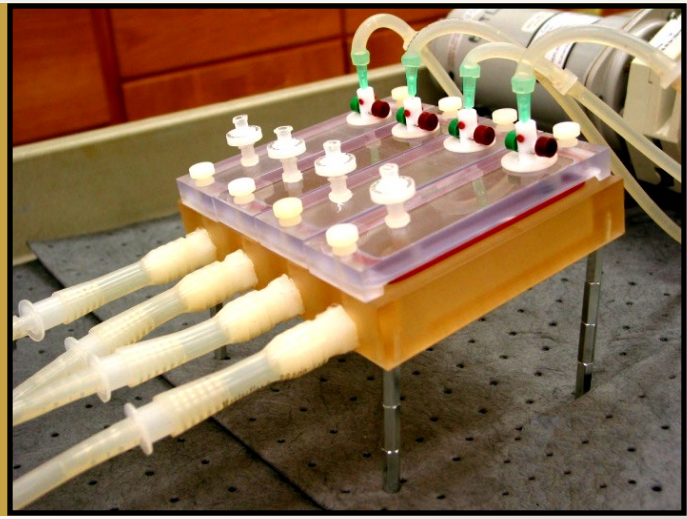
In vitro biofilm methods

Rotating Disk Reactor

Moderate shear
- Continuous flow -
ASTM E2196

Drip Flow Reactor (DFR)

Low shear
- Plug flow -
ASTM E2647

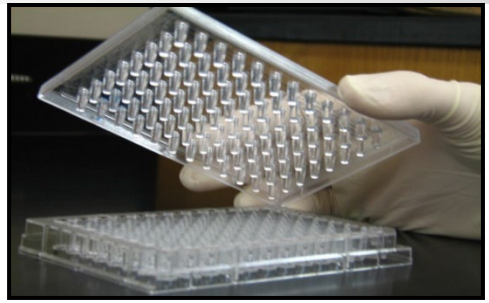


CDC Biofilm Reactor

High shear
- Continuous flow -
ASTM E2562

Single Tube Method (STM)

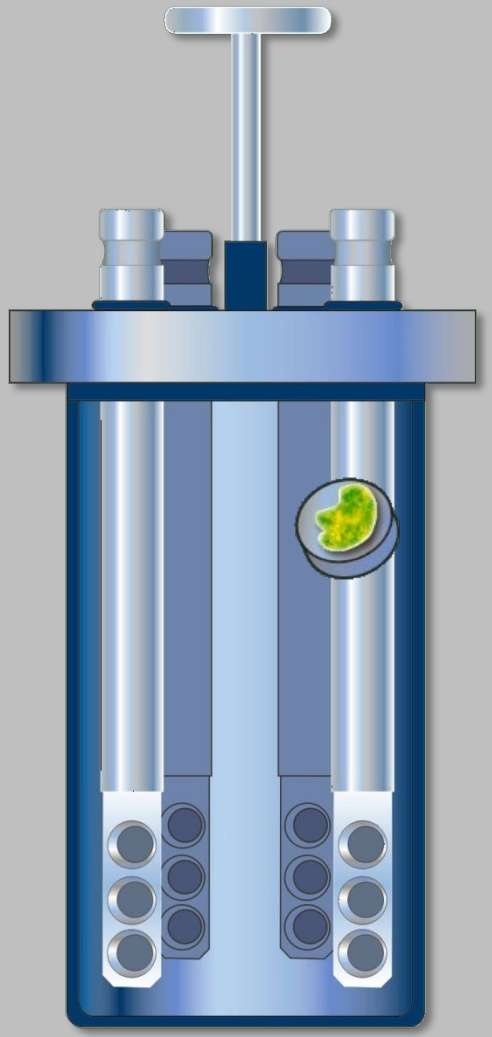
High shear
- Continuous flow -
ASTM E2871



MBEC Assay

Gentle shear
- Batch -
ASTM E2799

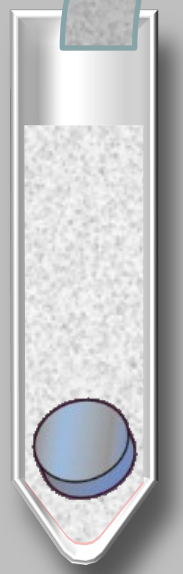
Single Tube Biofilm Method (STM)

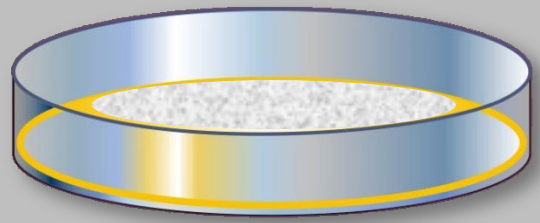


Disinfect

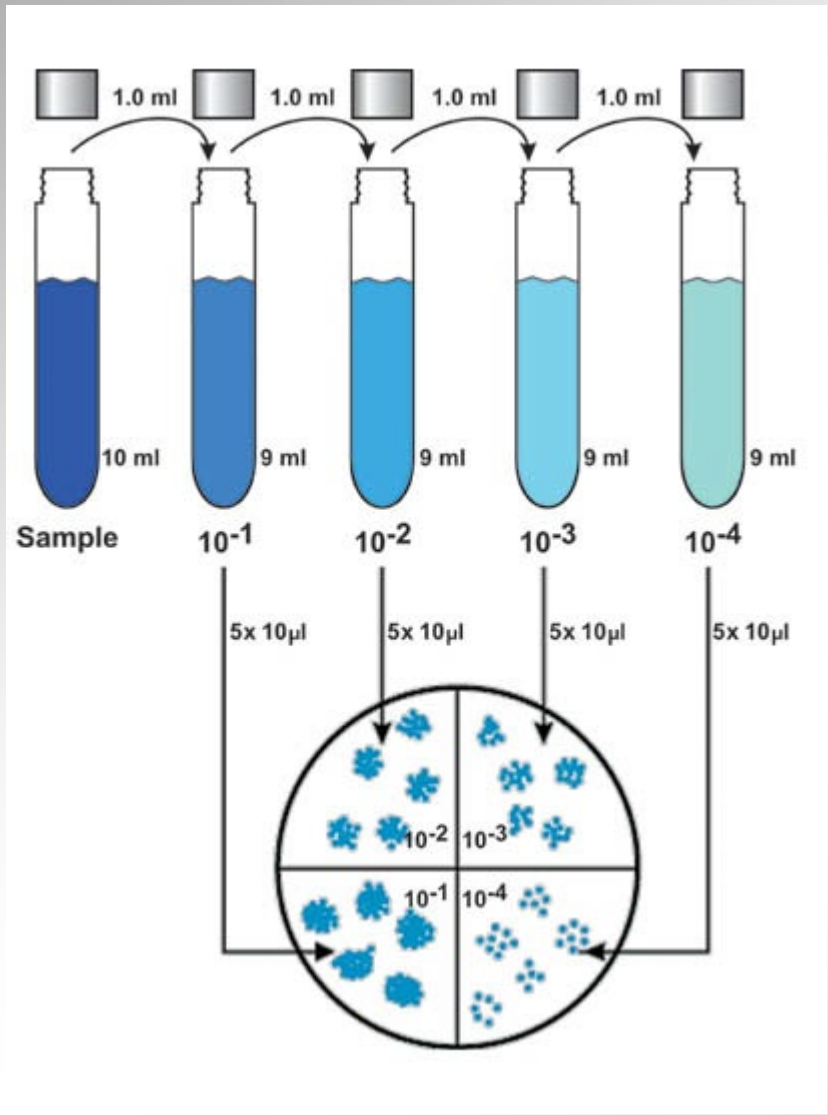
Neutralize

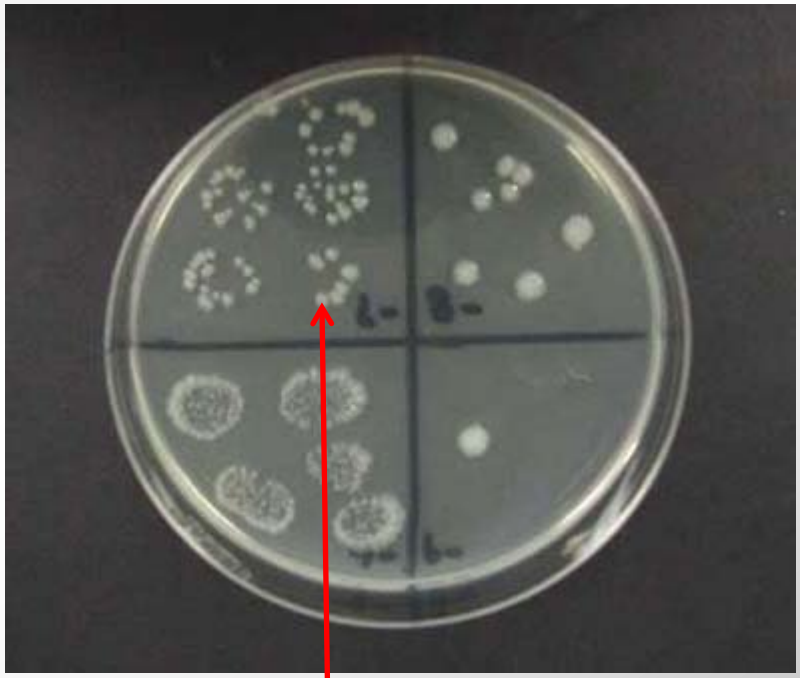
Disaggregate





Conventional way to quantify microbial abundances since 1880s

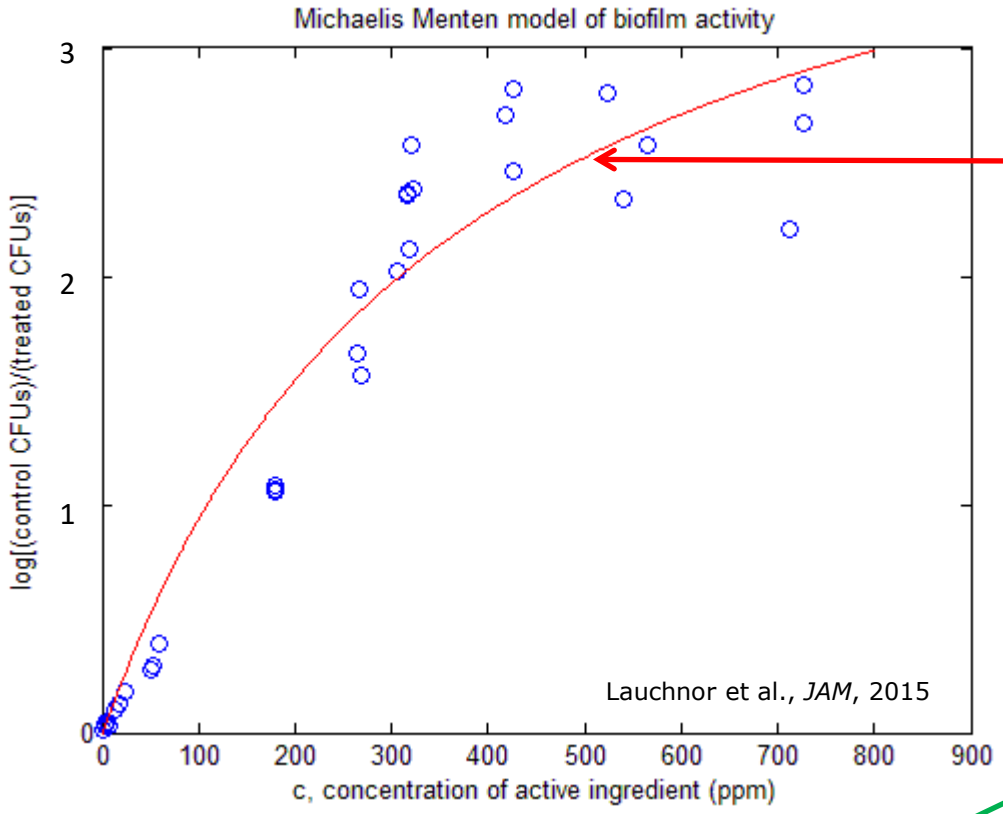




http://www.hypertextbookshop.com/biofilmbook/v004/r003/

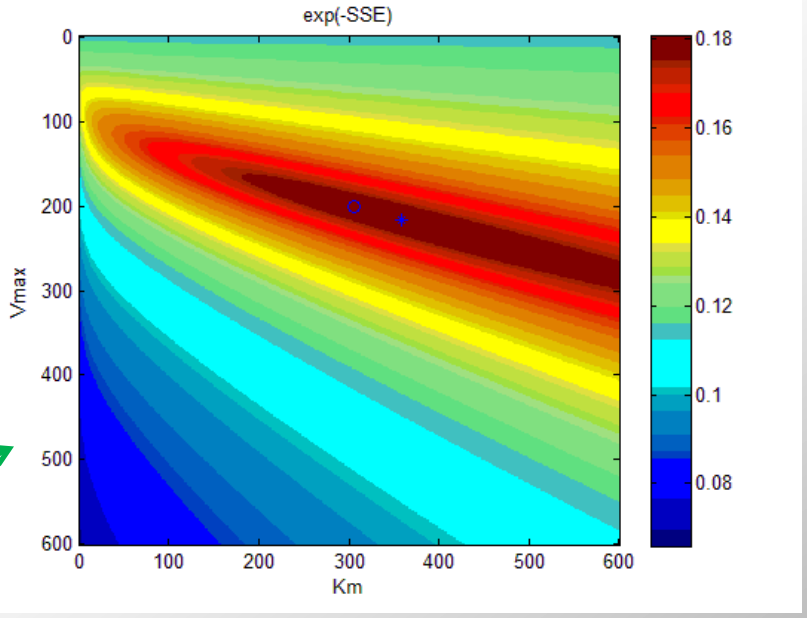
Colony forming units (CFU)
from 4 dilutions on an agar plate from a *P. aeruginosa* biofilm

Microbiologists in my lab usually want to kill biofilms:



Pick a model:
Michaelis-Menten/Monod,
lethality model + Gaussian noise:

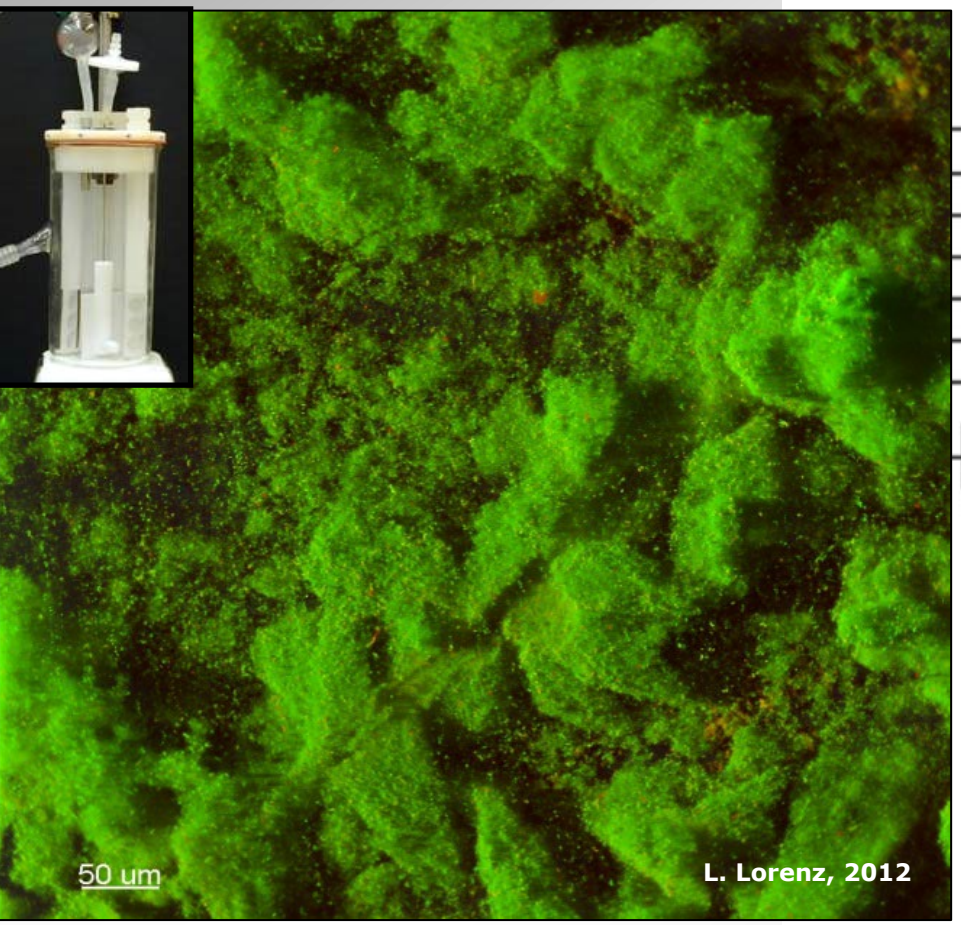
$$\Delta\text{abundance} = \log \text{reduction} = \frac{V_{max}c}{K_m + c} + \varepsilon$$



Inference via the posterior

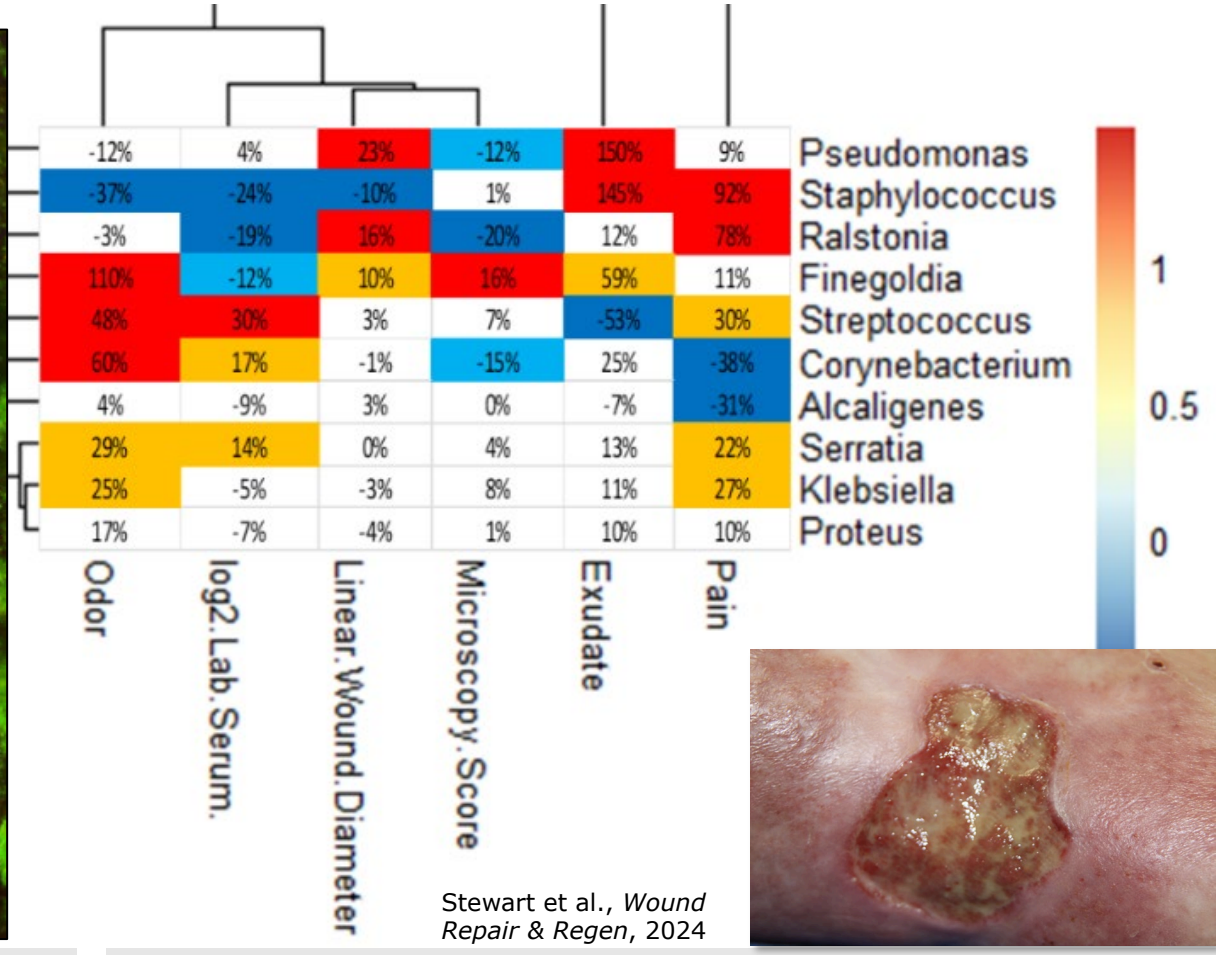
Modern Microbiological data

pixel intensities in an image



1024 x 1024 x 158 gray-scale image of stained *P. aeruginosa* biofilm

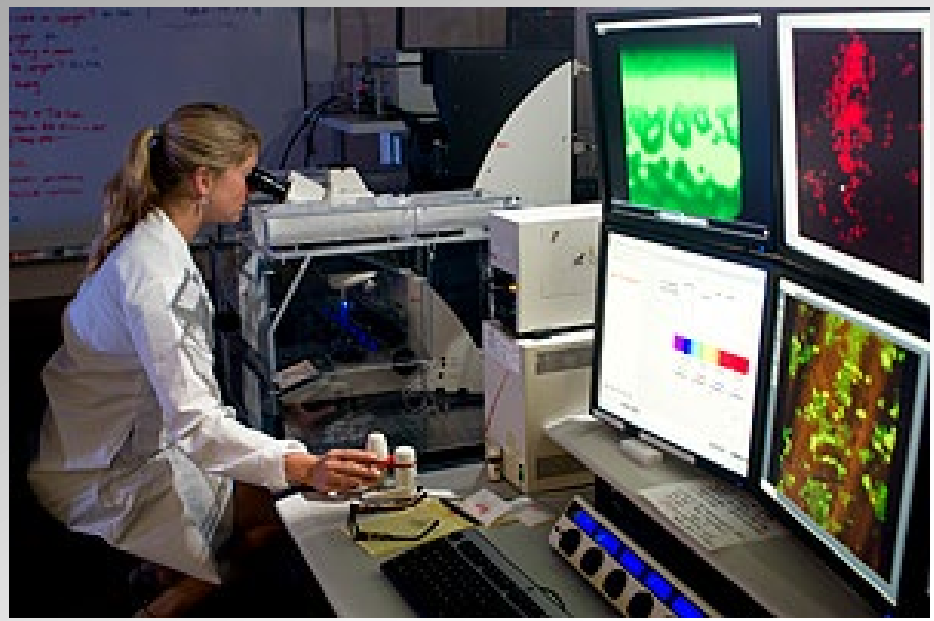
species detected by a molecular method

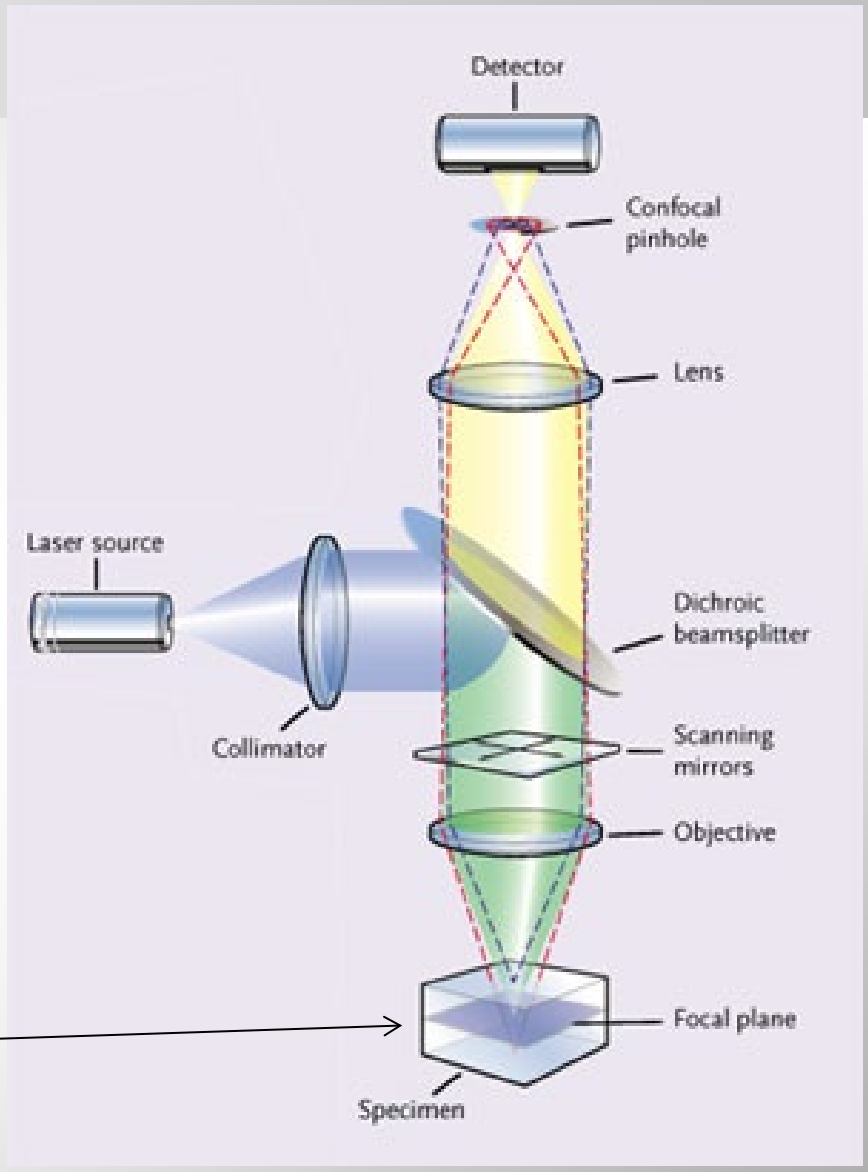


Stewart et al., *Wound Repair & Regen*, 2024

MiSeq data from 345 samples from chronic venous leg ulcers from 107 subjects over 8 weeks

Confocal Microscopy

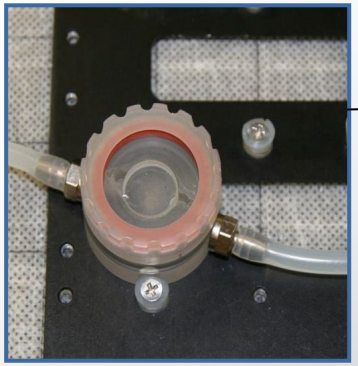




CDC Biofilm Reactor

High shear

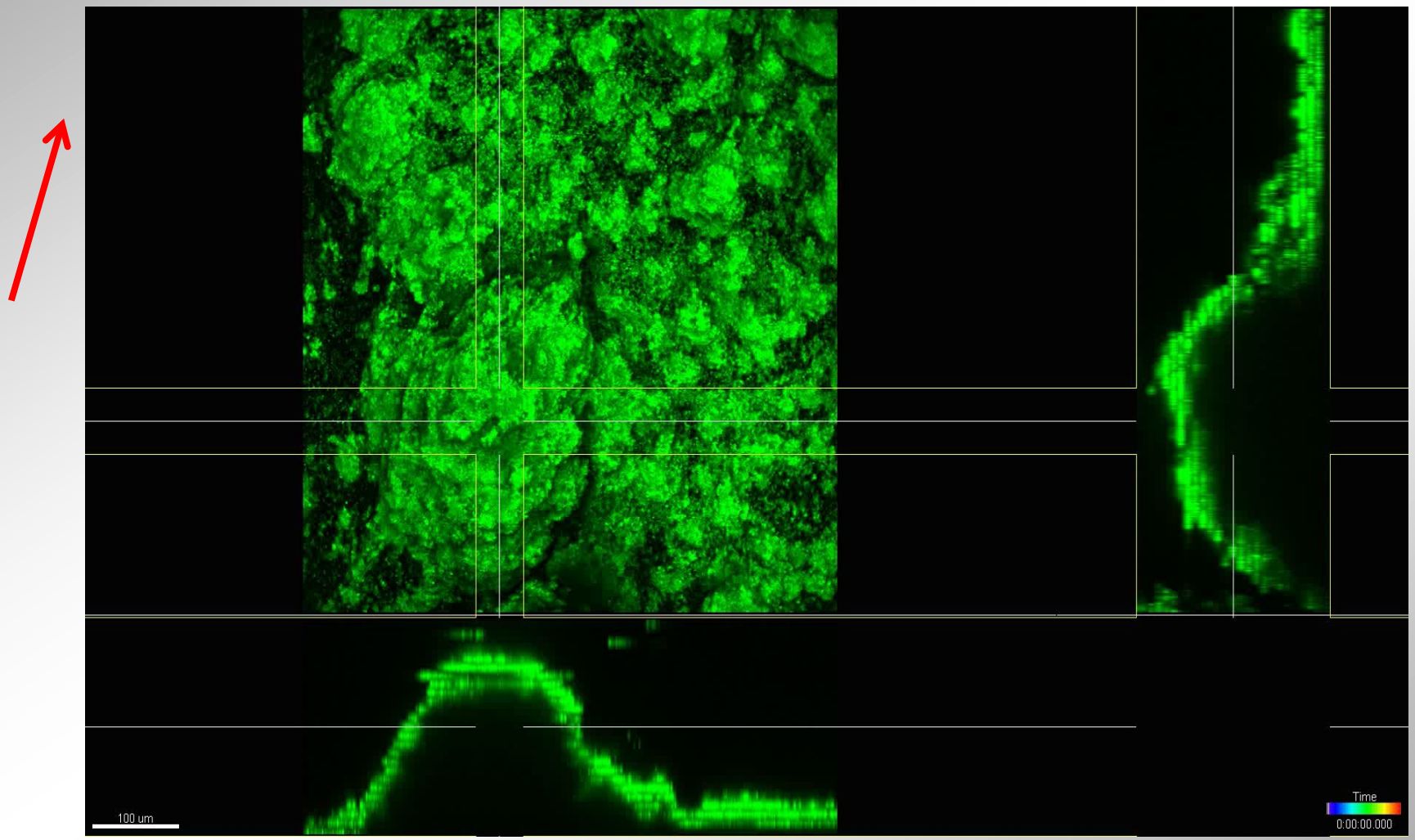
ASTM
Method E2562



Cartoon from A. Constans, The Confocal Microscope, *The Scientist*, November 22, 2004, http://www.the-scientist.com/?articles.view/articleNo/16043/title/The-Confocal-Microscope/

3D image data over time

- 512 x 512 x 60 gray scale image of a GFP *S. aureus* biofilm
- 4 frames/minute over an hour

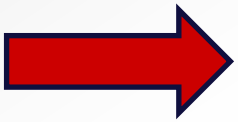


Common questions addressed by biofilm image analysis:

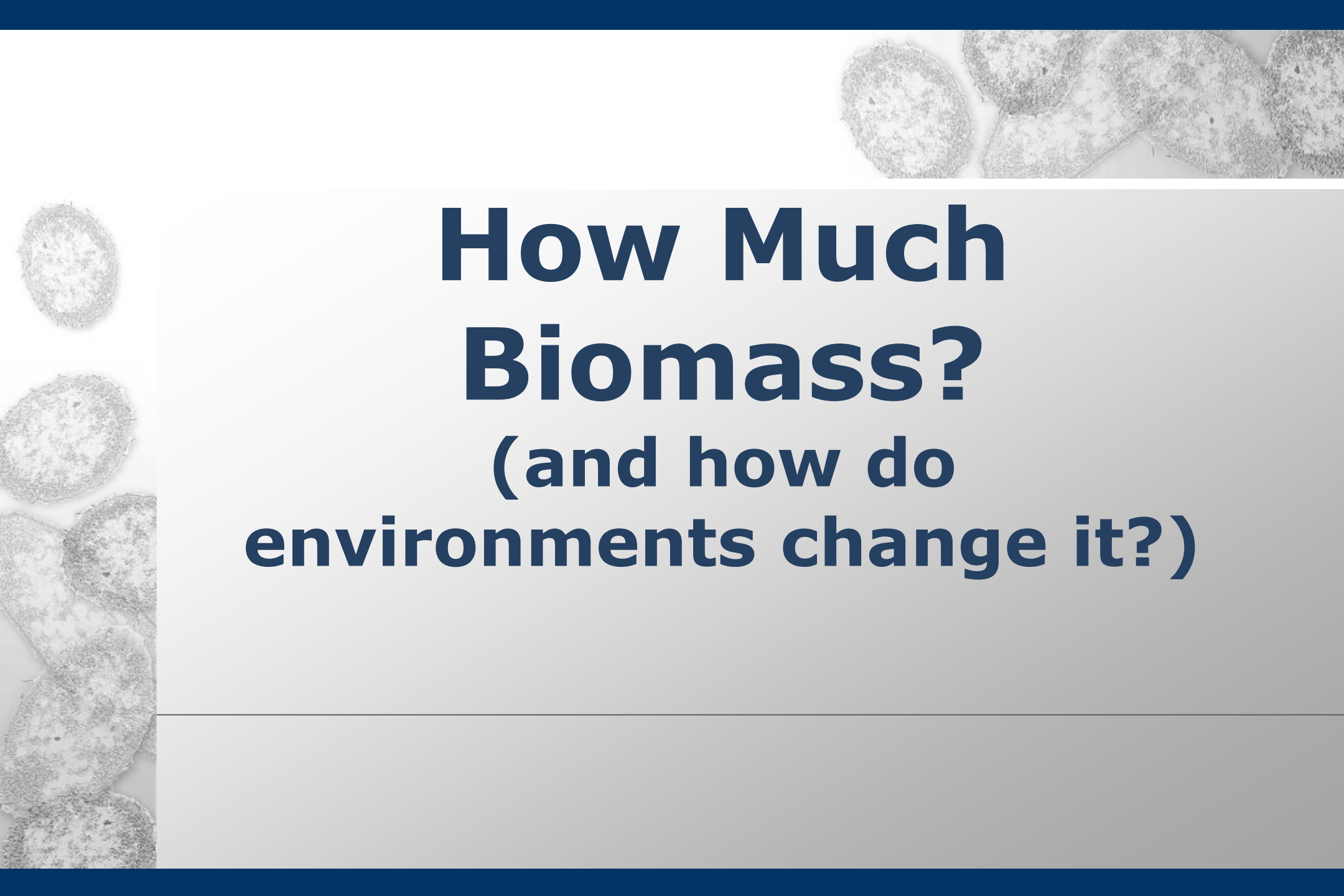
- How much biomass?
- Where are the microbial cells?
- Is there any biomass?
- How do multiple species correlate in space?

Practical Questions:

- How much variability is there?
- How to perform calculations efficiently?

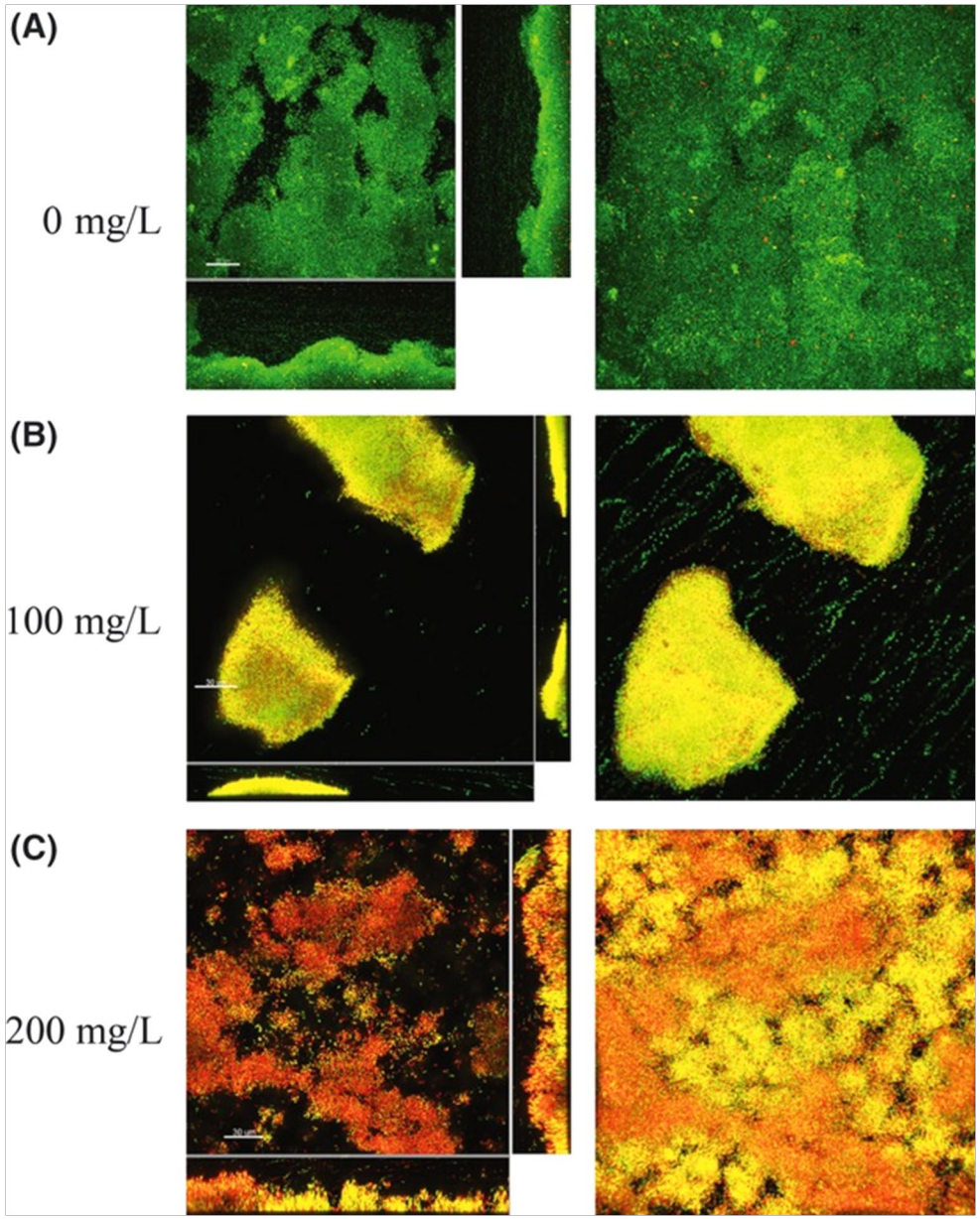


A Biofilm Imaging Library Is Needed!



How Much Biomass?
(and how do environments change it?)

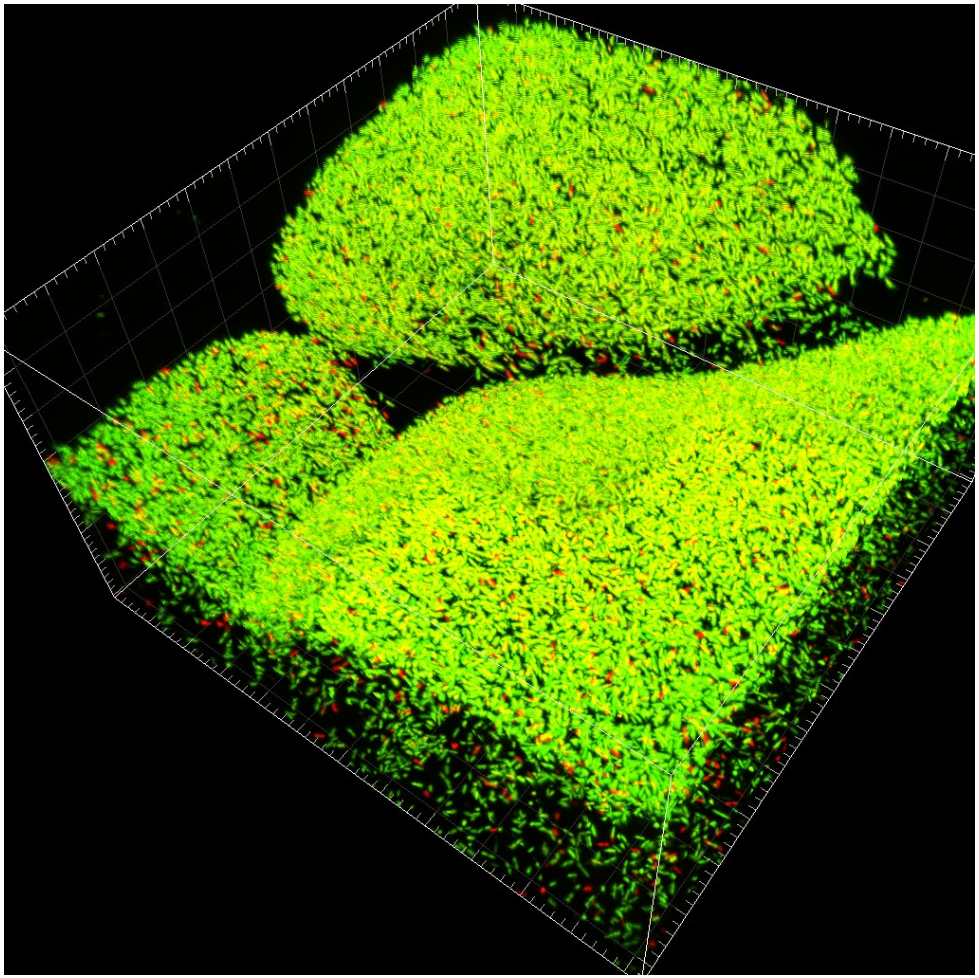
Cationic steroid antibiotics applied to *Pseudomonas*

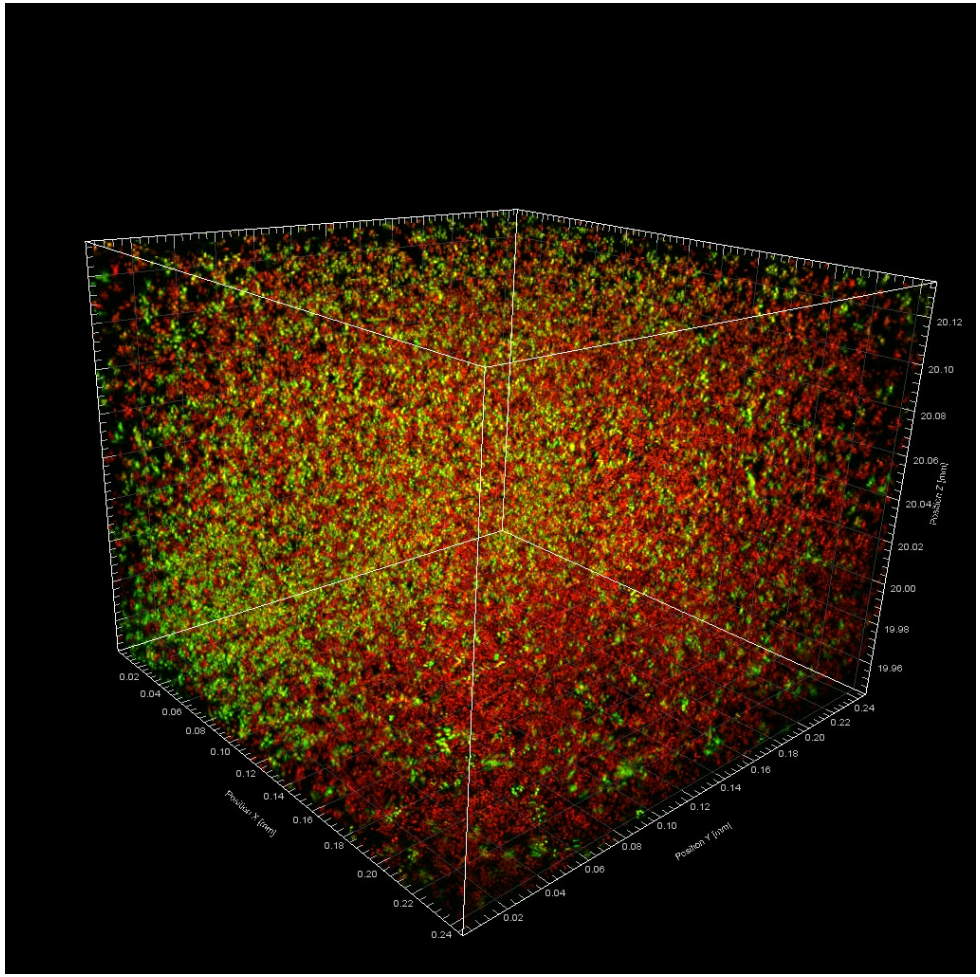


grown in CDC reactor,
Live/Dead stained

Nagant *et al*, *MicrobiologyOpen*, 2013

Hydrogen peroxide applied to Antarctic *Janthinobacterium*





control

grown in CDC reactor, Live/Dead stained
FOV: 620um x 620um x 141um
Pixilation: 1024 x 1024 x 286

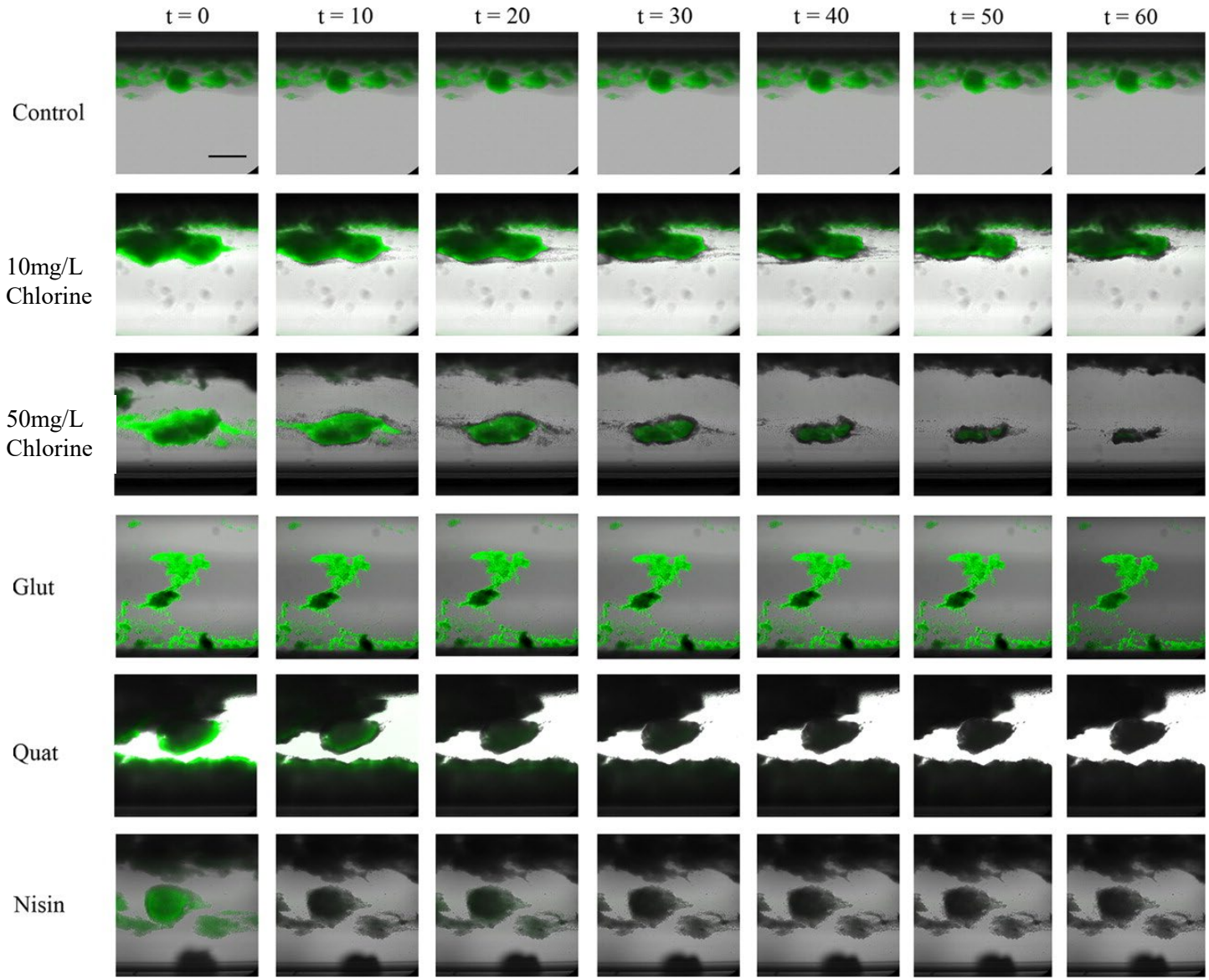
treated

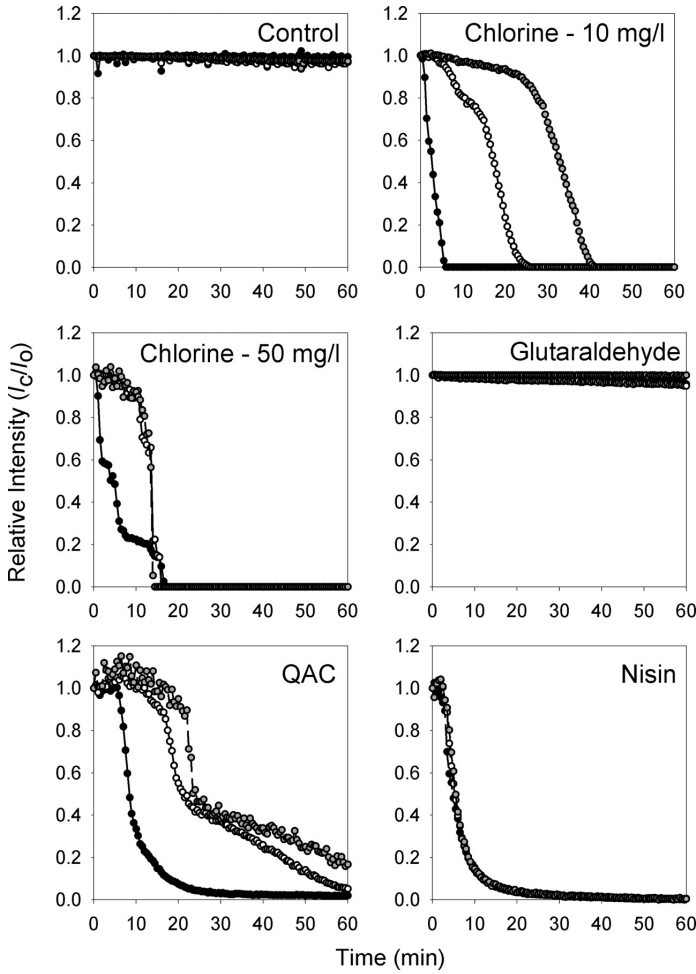
Smith *et al*, *Genome Announce*, 2016

Three old-school global biomass quantitation from 3D images:

- **average intensity**
- **biovolume** = volume of bright pixels
  (pixels with intensities above a threshold)
- **average thickness** = biovolume/FOV
- **surface coverage (%)**

Staph epi average intensities over time

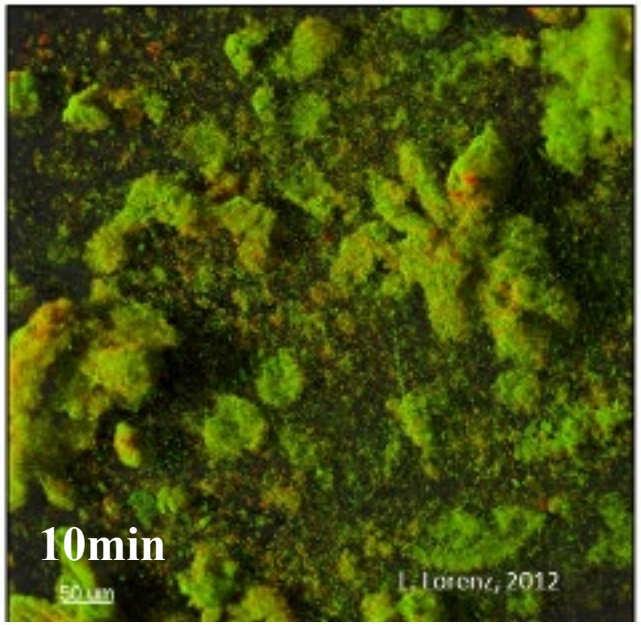


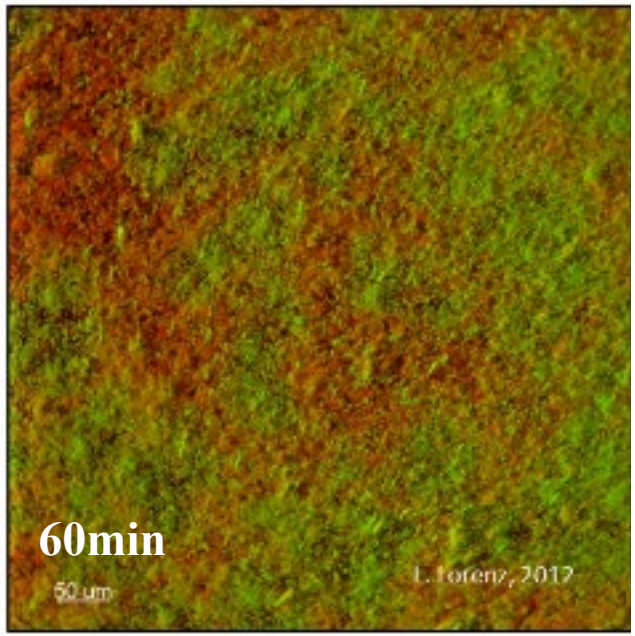


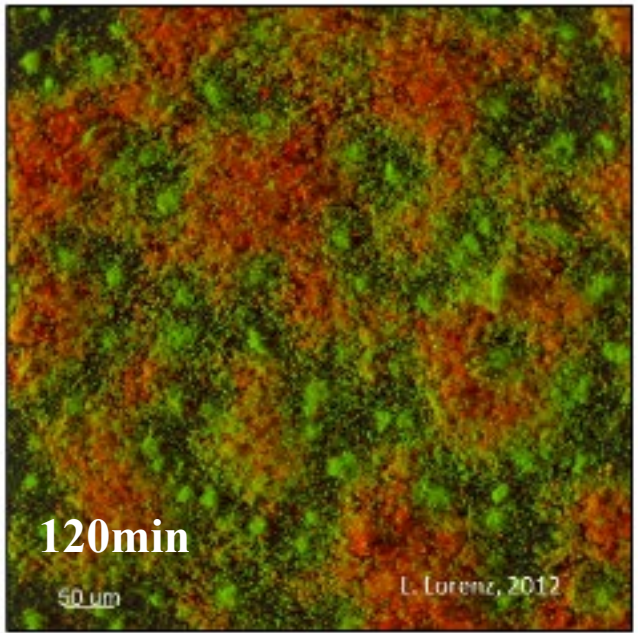
grown and imaged in glass capillary reactor, calcein-AM stained
2 images/minute over an hour

Davison et al,
Antimicrobial Agents Chemo, 2010

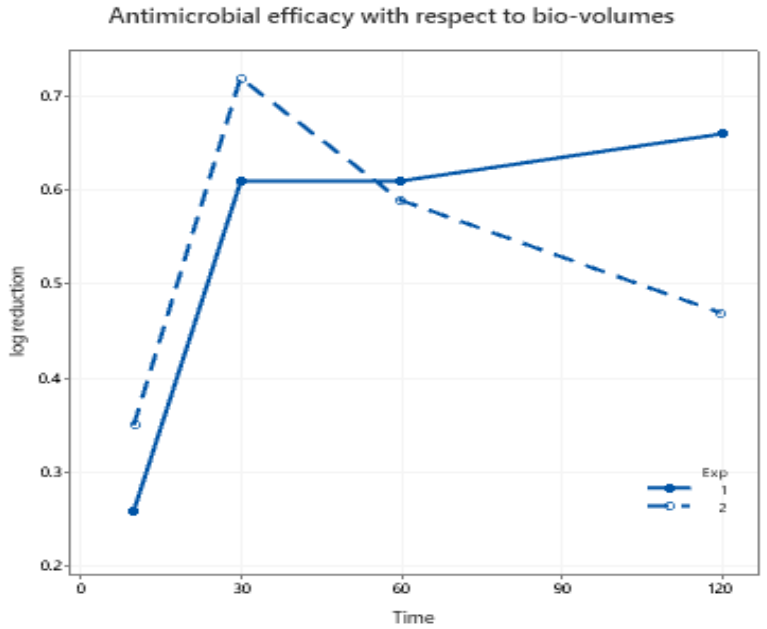
Pseudomonas biovolumes over time





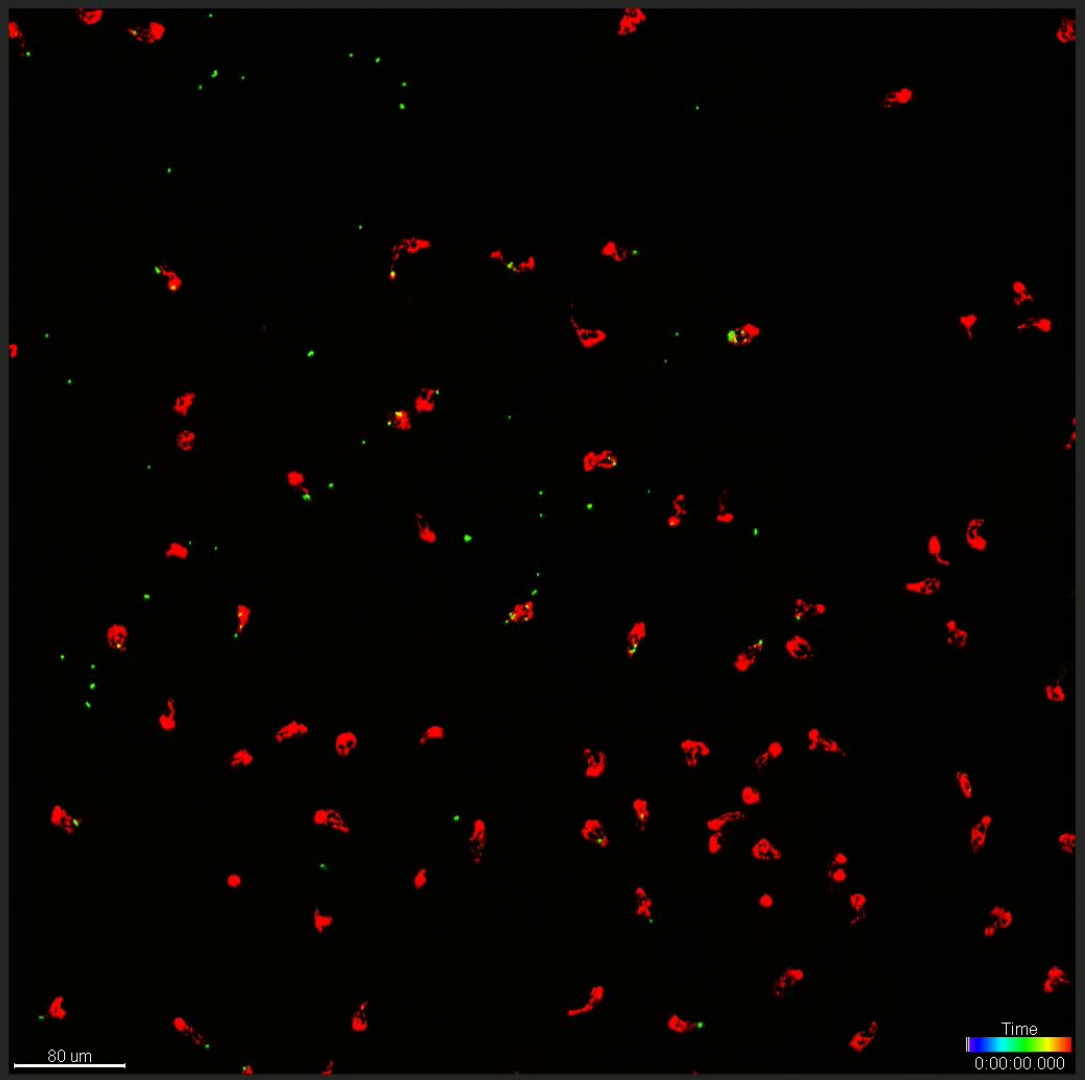


grown in CDC reactor, Live/Dead stained
Treatment: Photoactivated chlorine dioxide
FOV: 620um x 620um x (up to 260)um
Pixilation: 1024 x 1024 x 100s

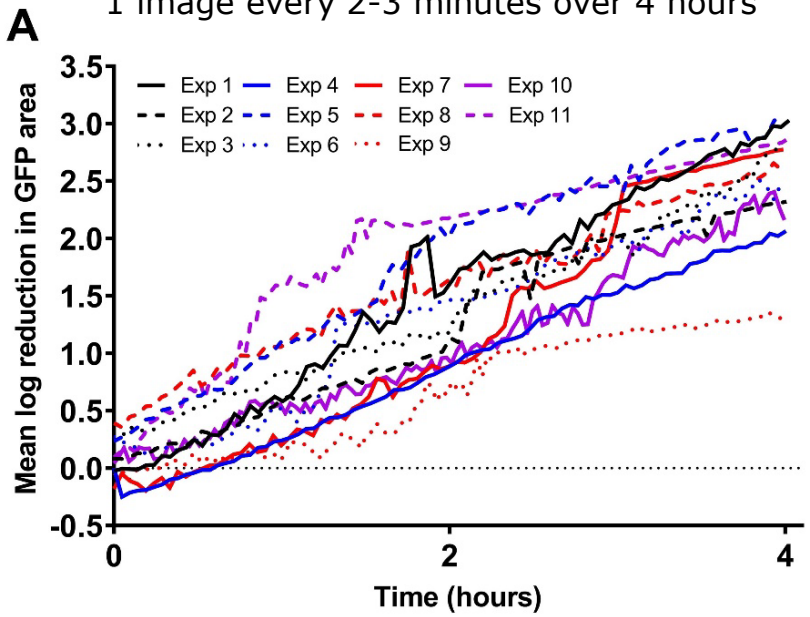


Parker *et al*, *J Applied Micro*, 2022

Staph aureus surface coverage over time

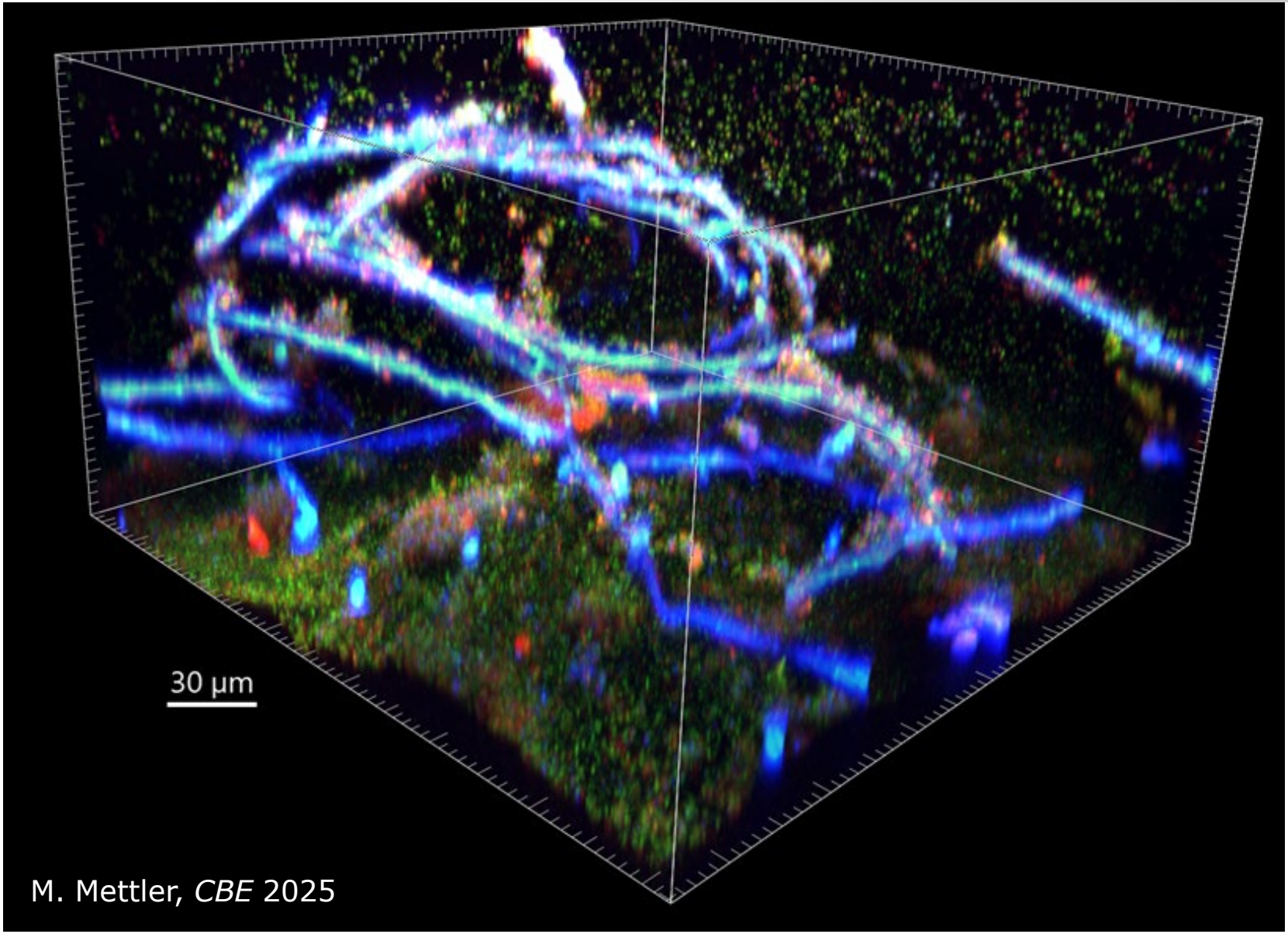


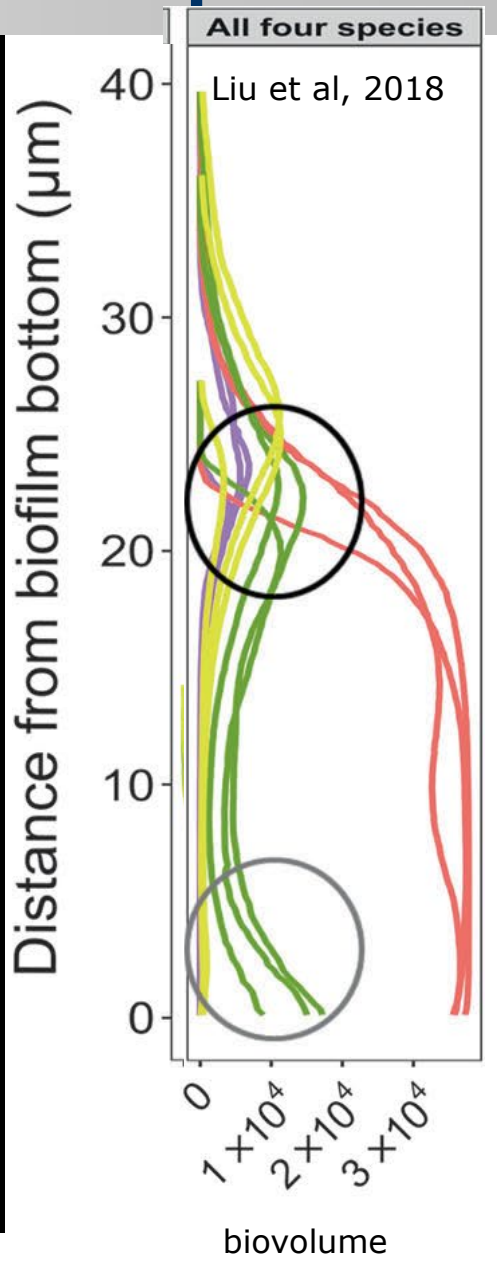
cultured overnight,
GFP Staph, red stained neutrophils
Treatment: Neutrophils
FOV: 620um x 620um x 20um
Pixilation: 512 x 512 x 20
1 image every 2-3 minutes over 4 hours



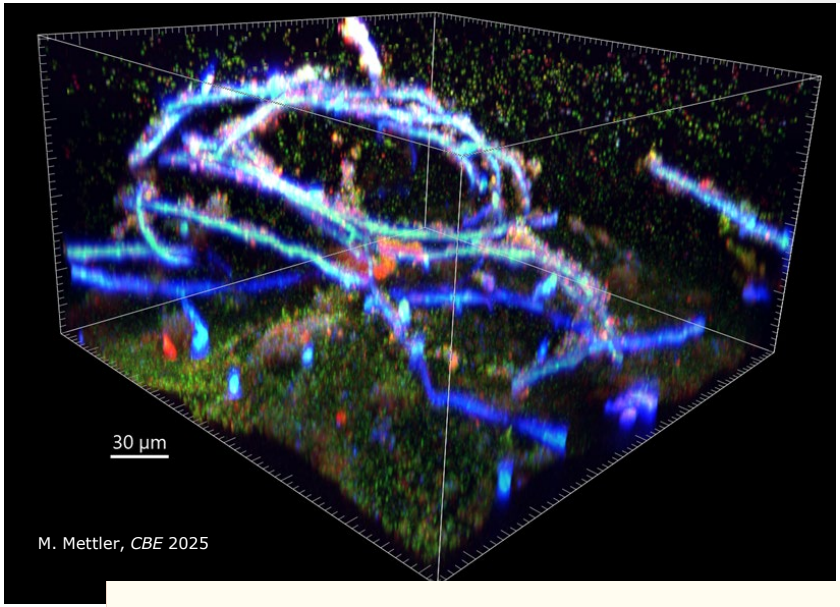
Pettygrove et al, *Biomaterials*, 2021

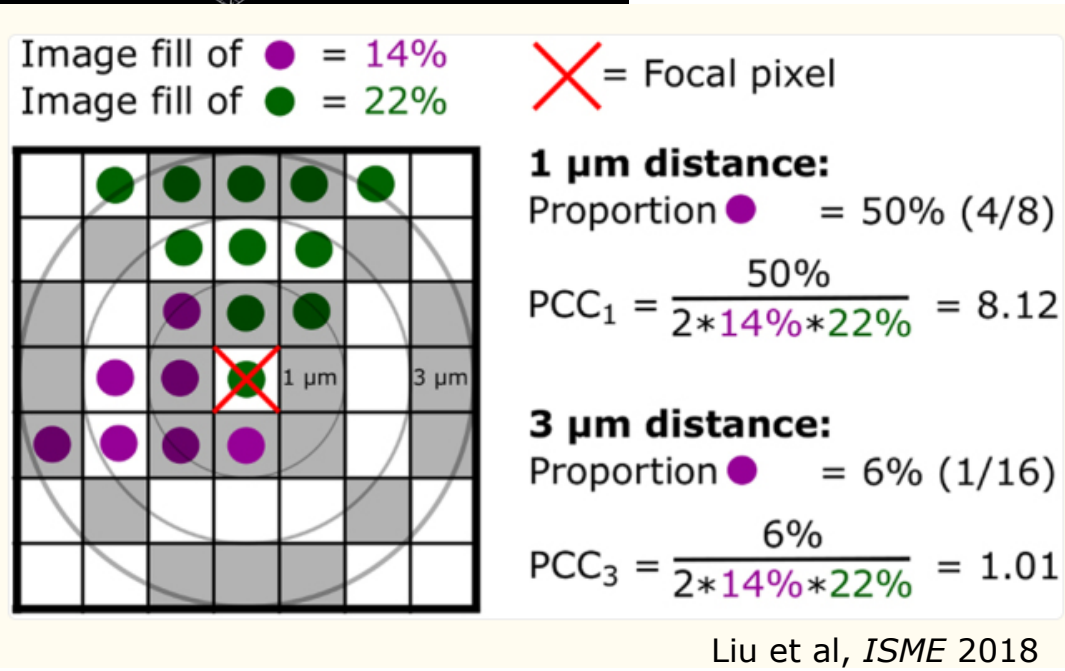
Multi-species / multi-kingdom biovolume over depth



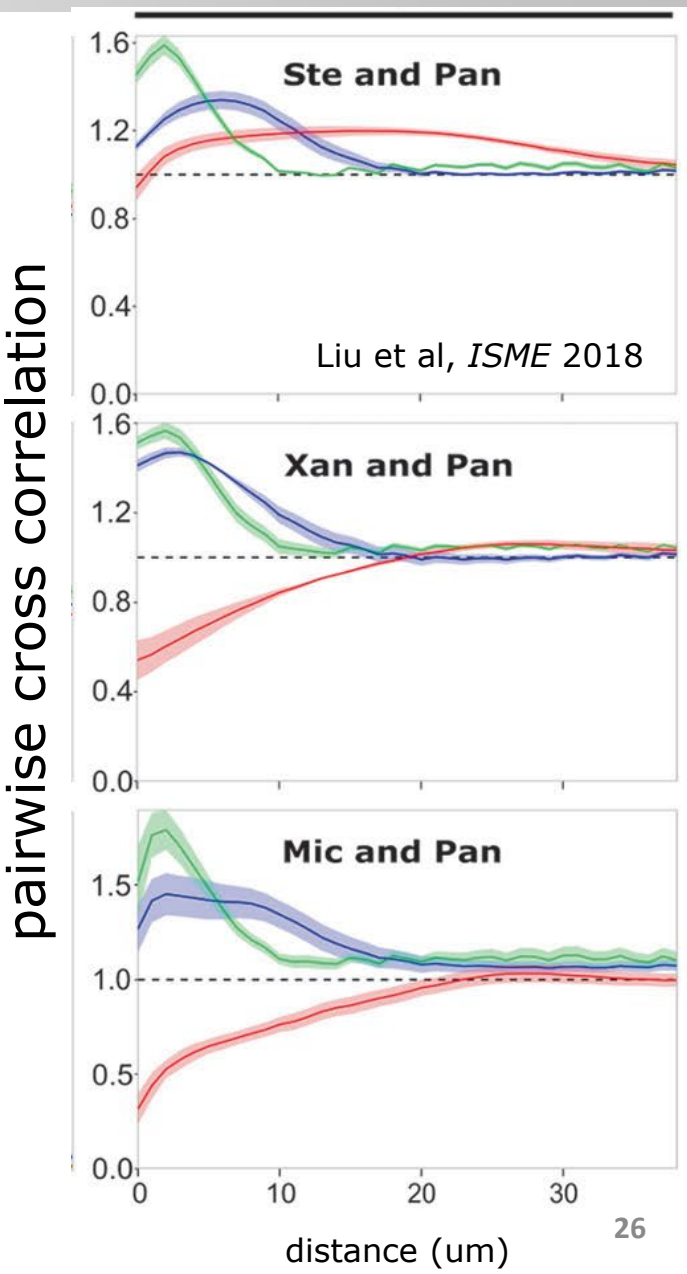


Quantifying Species Colocalization is Crucial yet Rarely Done



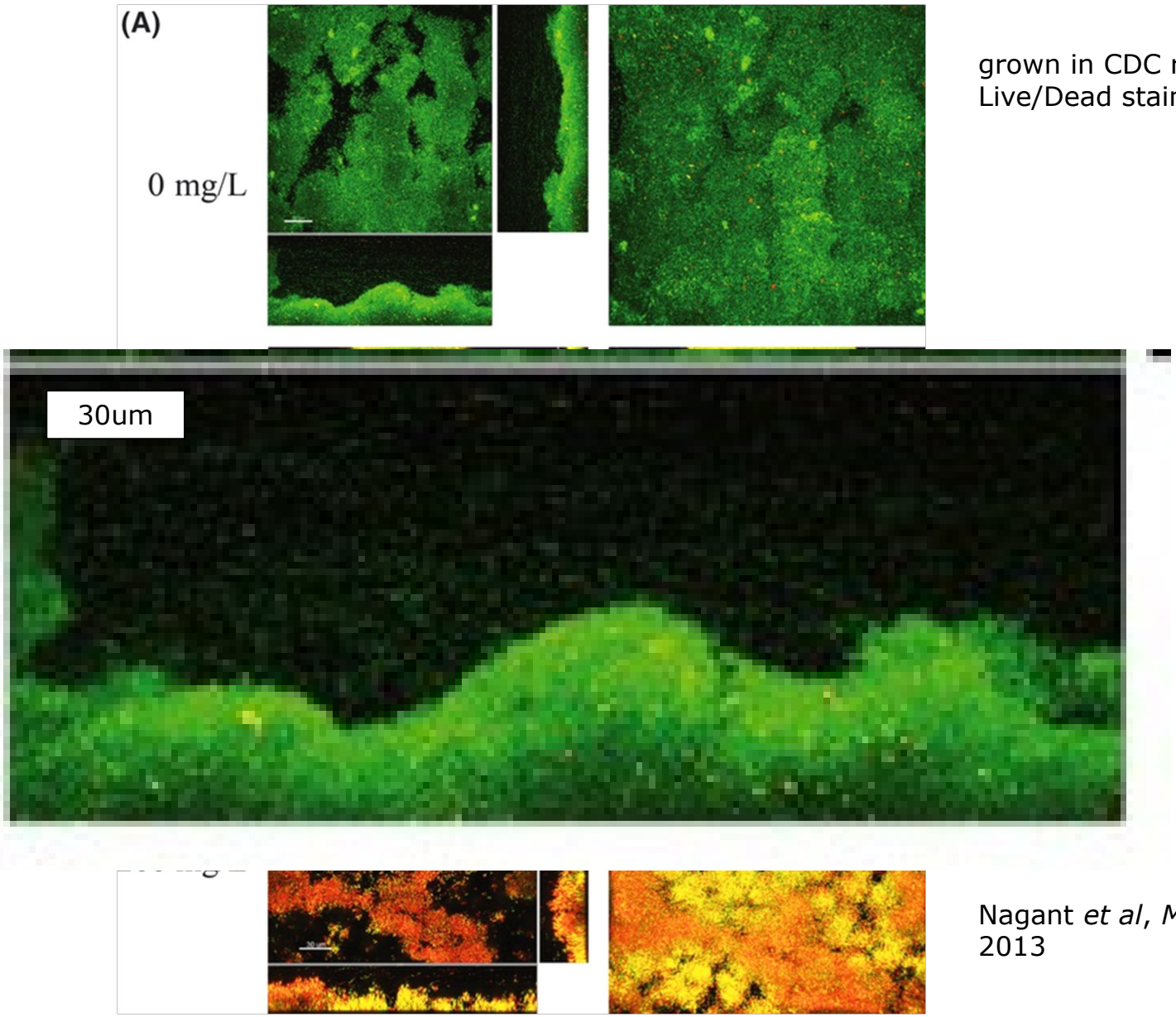


Liu et al, *ISME* 2018



Biofilm Thickness affects Image Intensity and Biovolume Calculations

Cationic steroid antibiotics applied to *Pseudomonas*

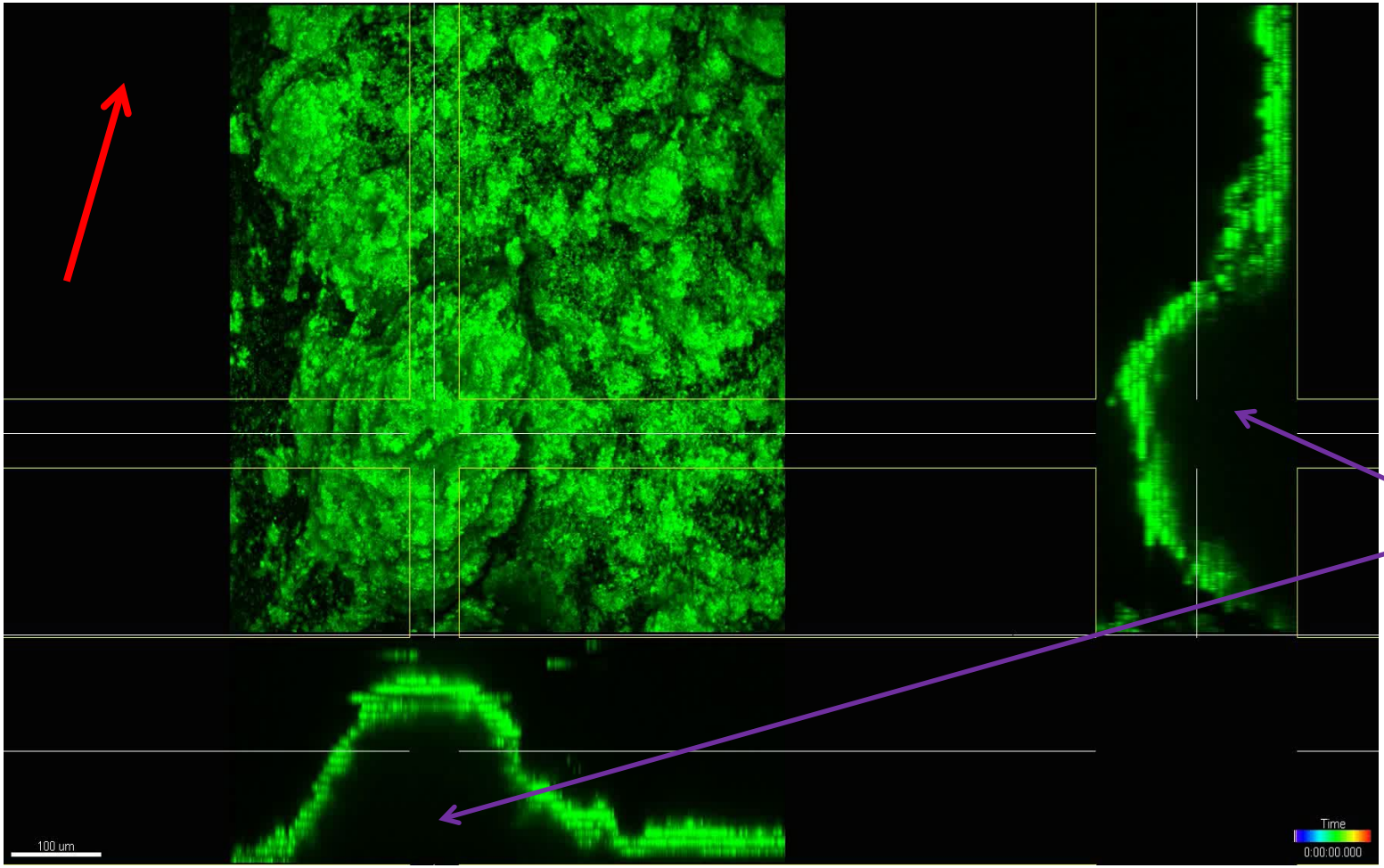


grown in CDC reactor,
Live/Dead stained

Nagant *et al*, *MicrobiologyOpen*, 2013

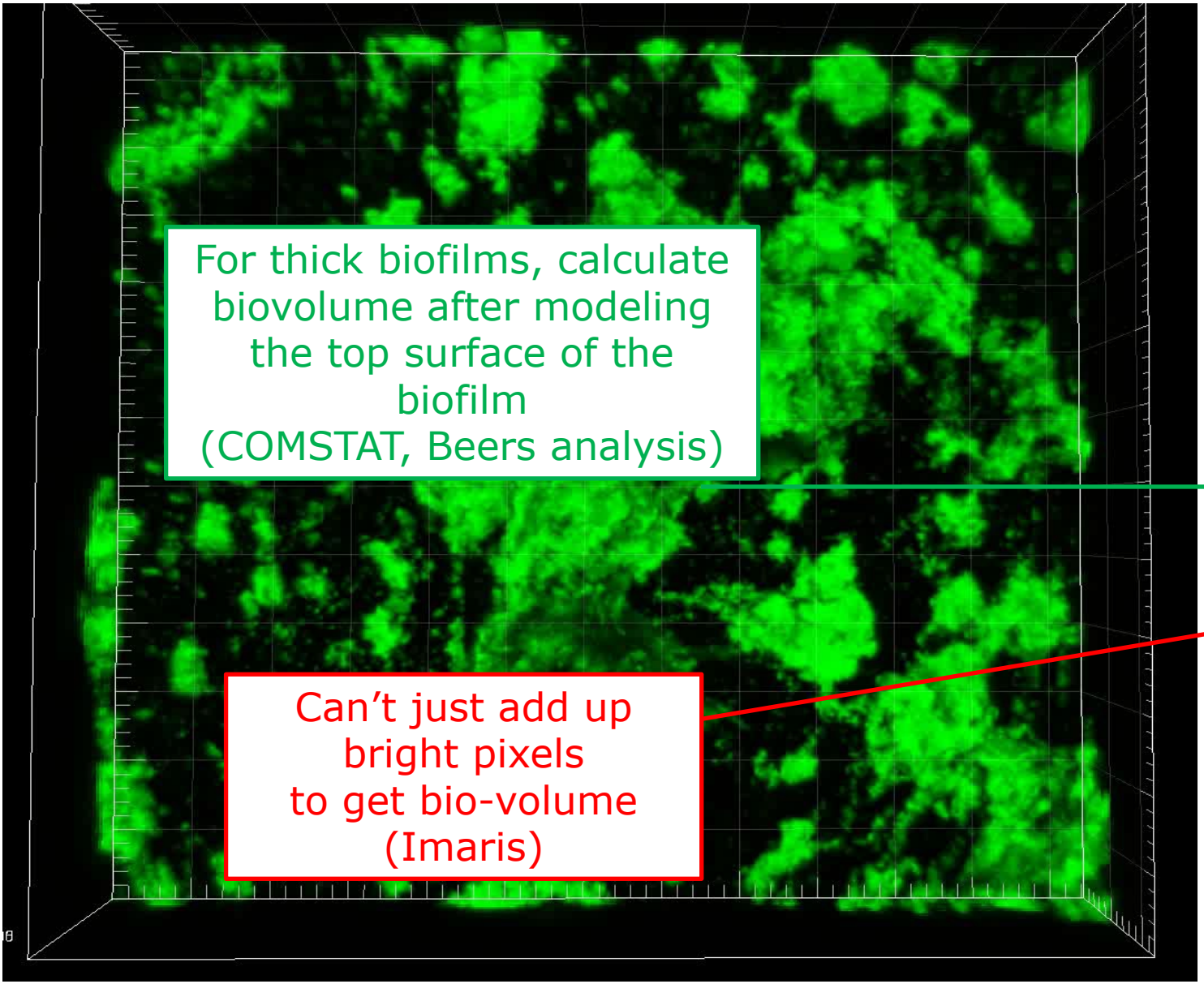
Microbiological data over time

- 512 x 512 x 60 gray scale image of a GFP *S. aureus* biofilm
- 4 frames/minute over an hour

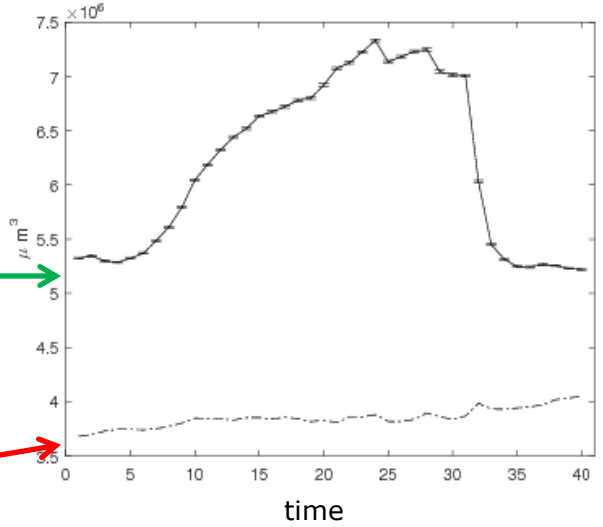


Drastic light attenuation deep in the biofilm is due to Beer's Law

Salt water treatment applied to *Staph aureus*

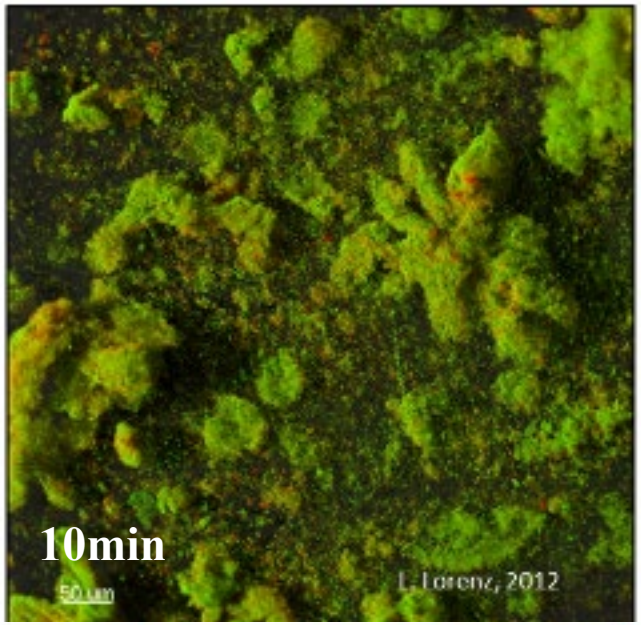


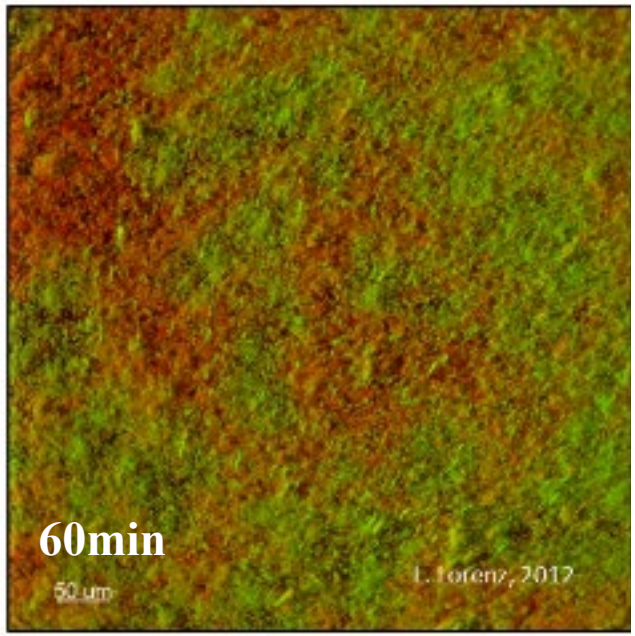
GFP
FOV: 620um x 620um x 112um
Pixilation: 512 x 512 x 17
4 images/minute over 45 minutes

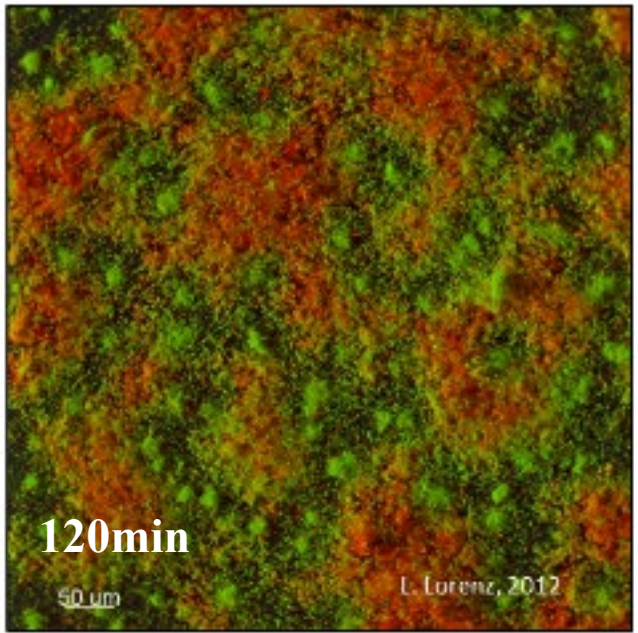


Parker et al, *J Am Stat Assoc*, 2018

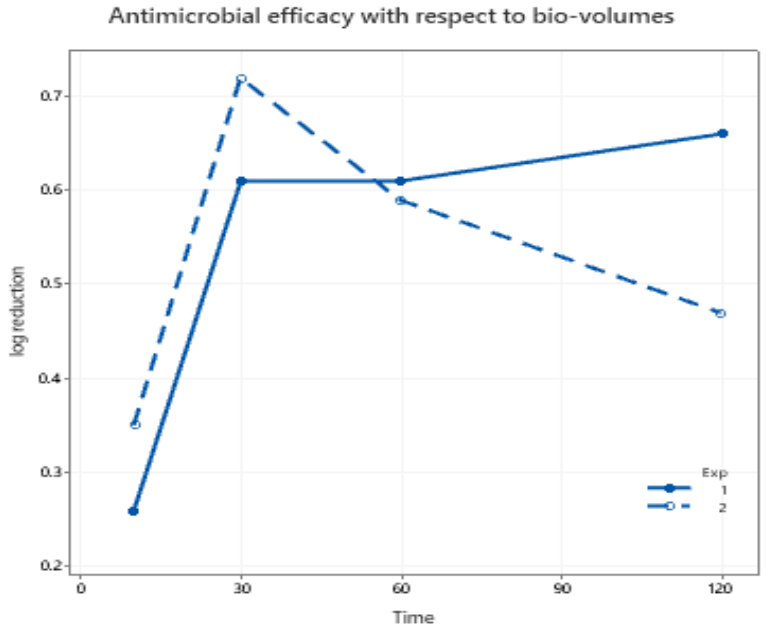
Photoactivated chlorine dioxide applied to *Pseudomonas*





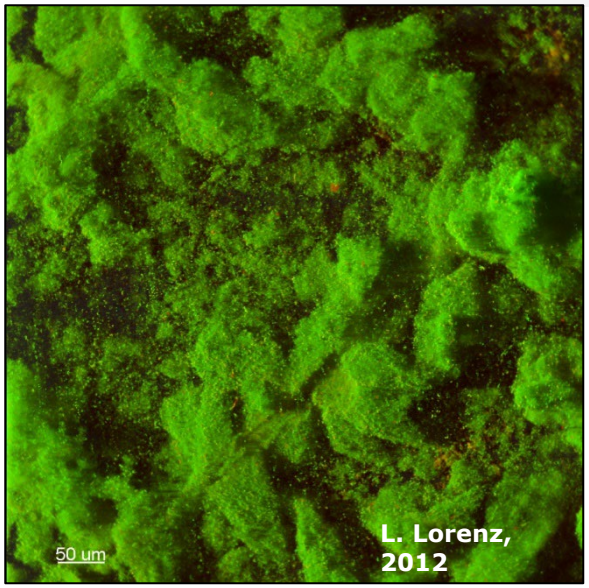


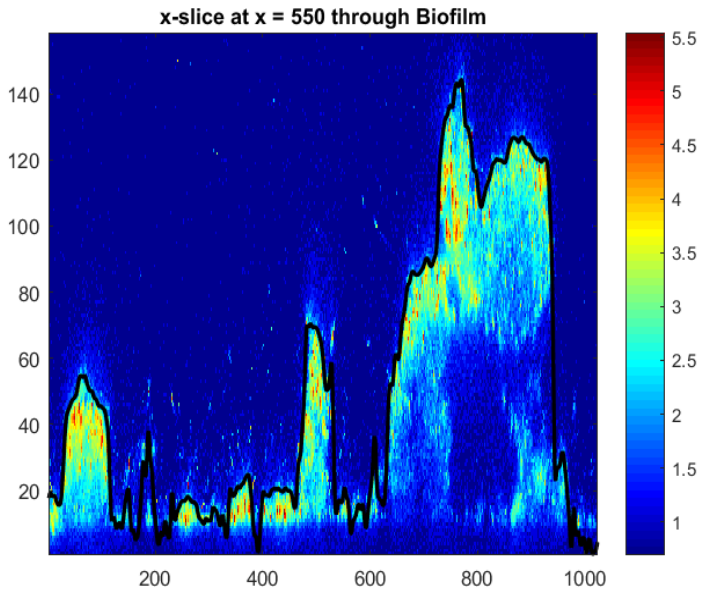
grown in CDC reactor, Live/Dead stained
FOV: 620um x 620um x (up to 260)um
Pixilation: 1024 x 1024 x 100s

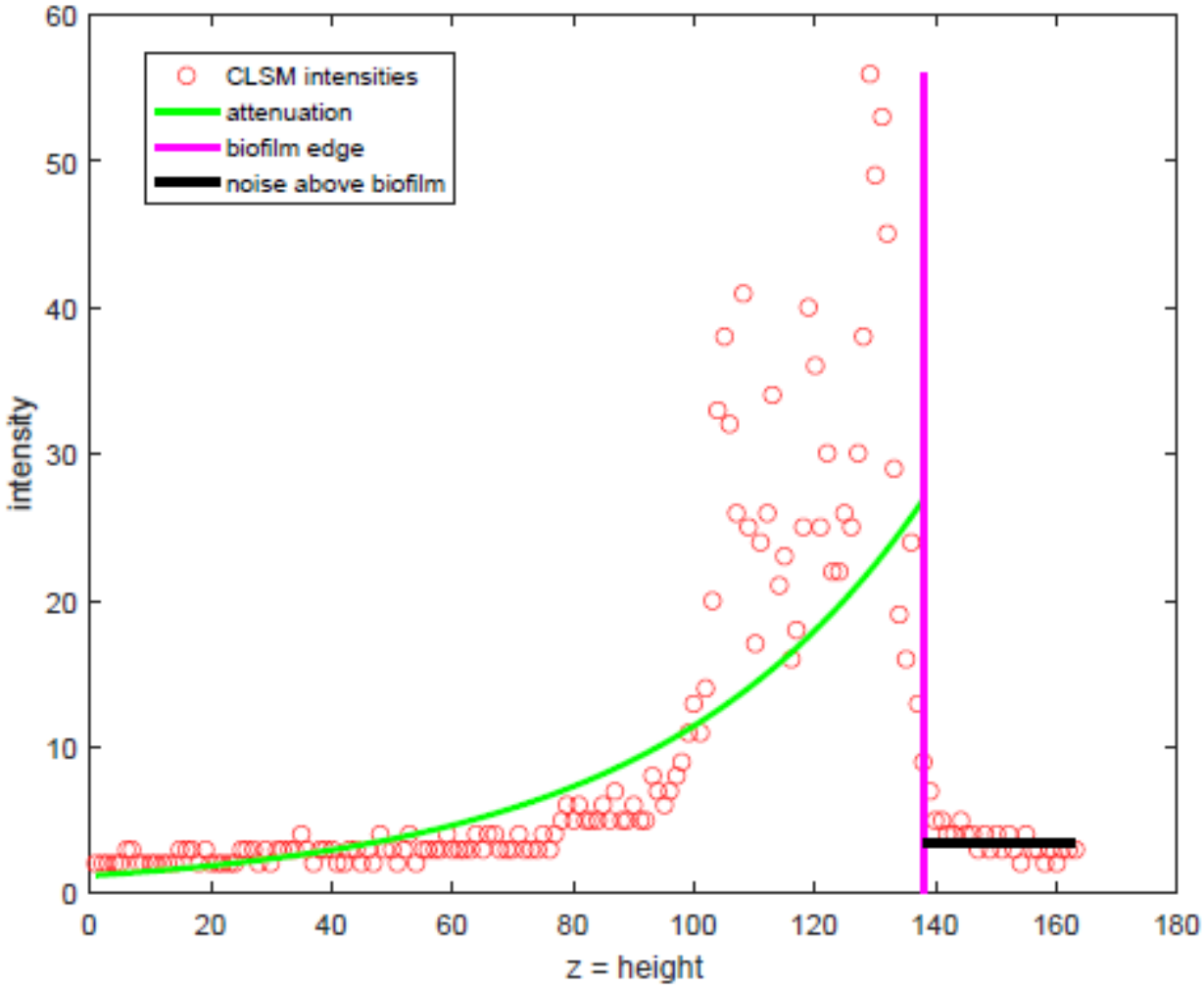


Parker *et al*, *J Applied Micro*, 2022

The attenuation can be modeled using Beer's Law

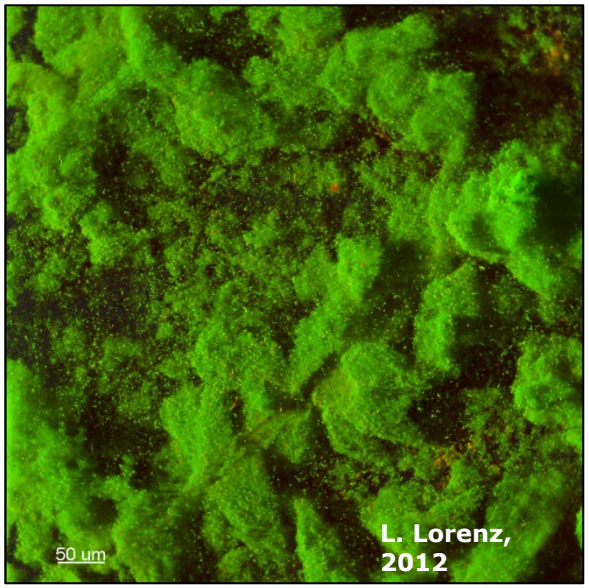


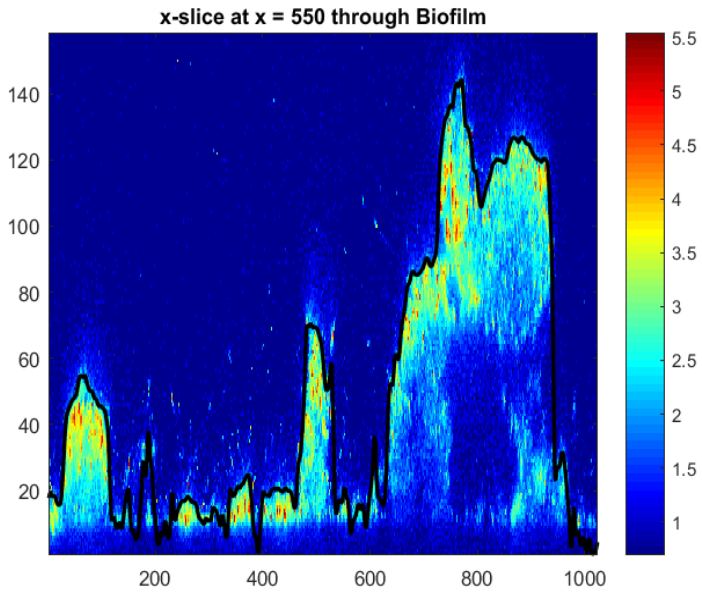




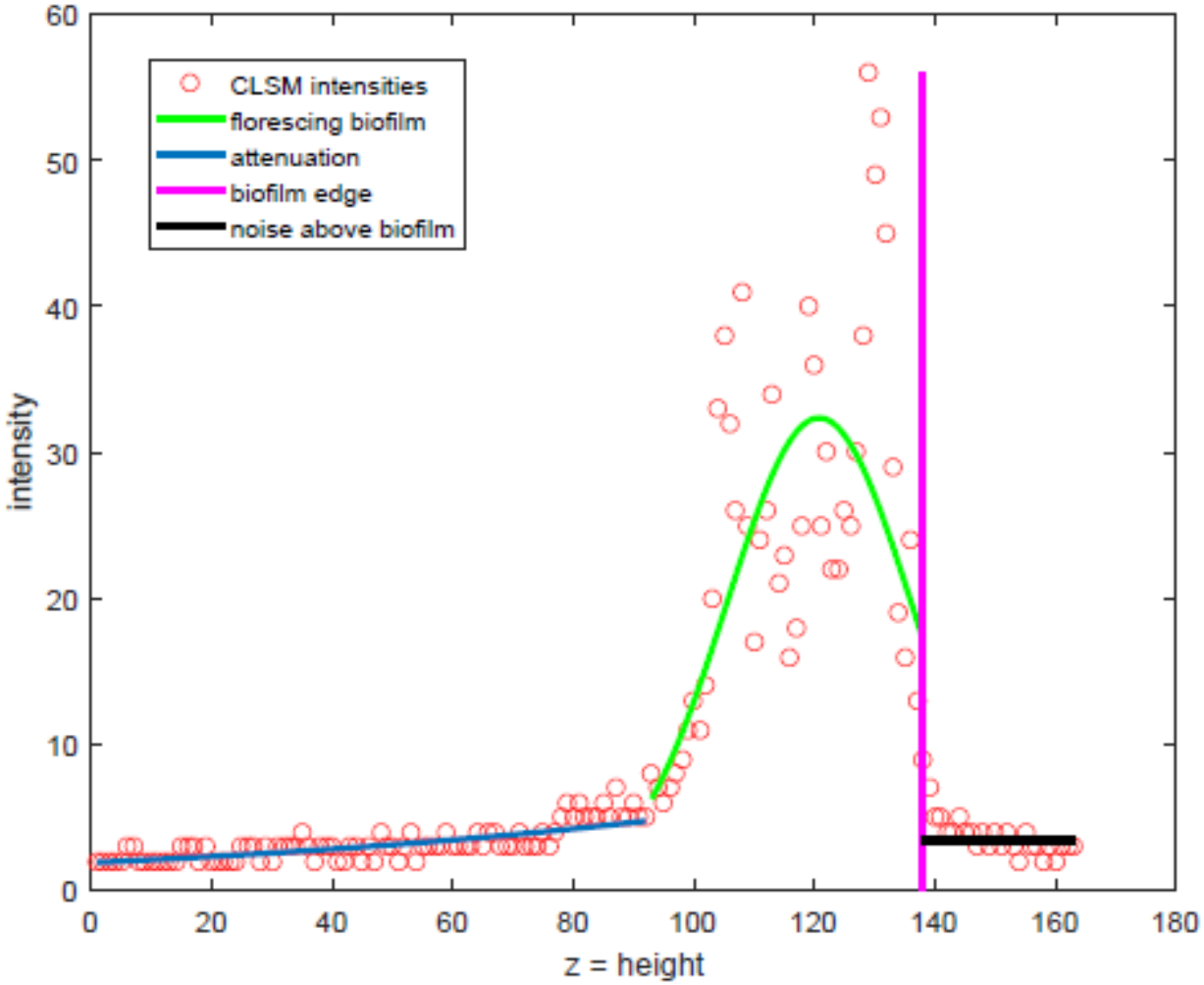
Model attenuation in each column of the image

Need a slightly more complicated model of attenuation



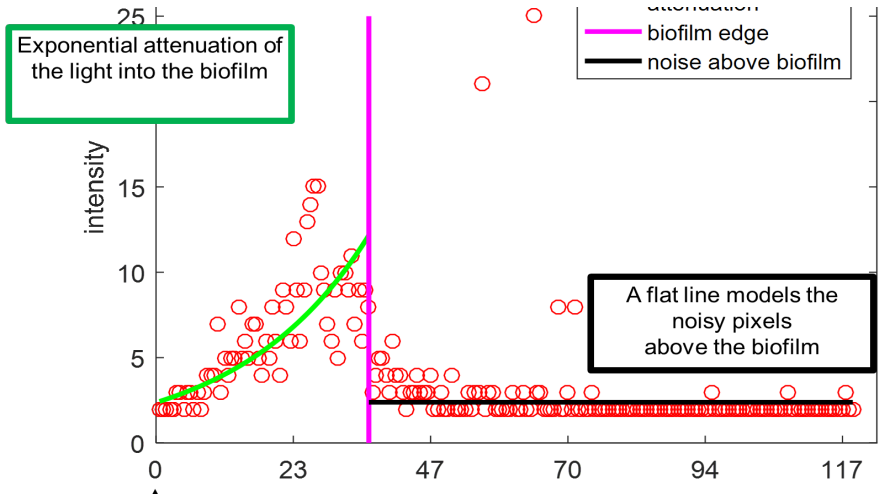


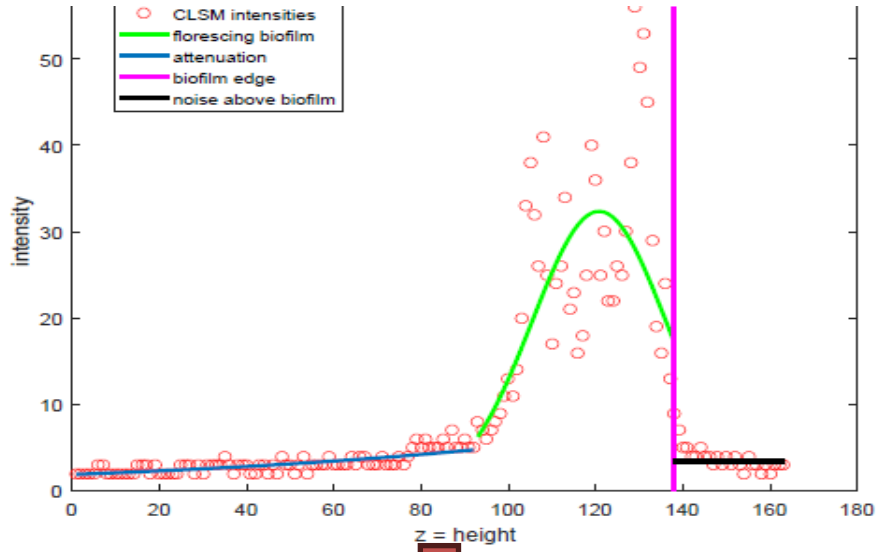
Parker et al, *J Micro Methods*, 2020

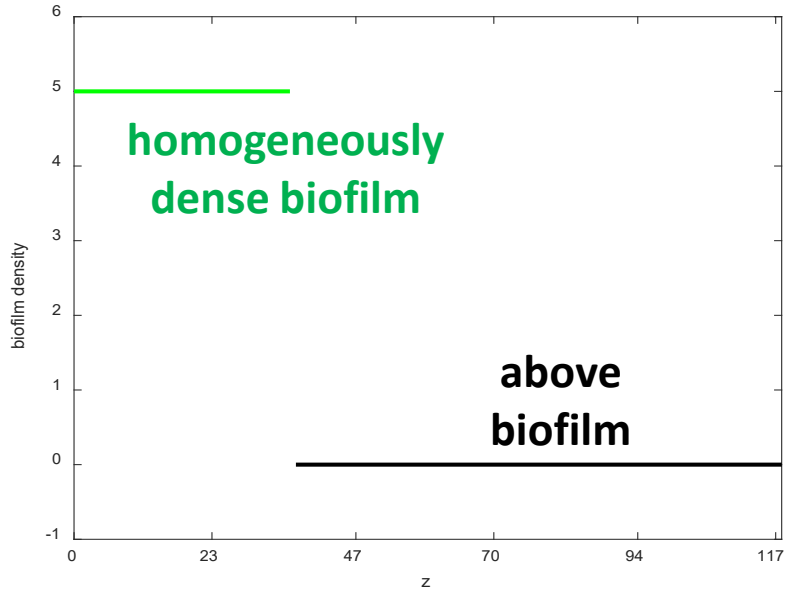


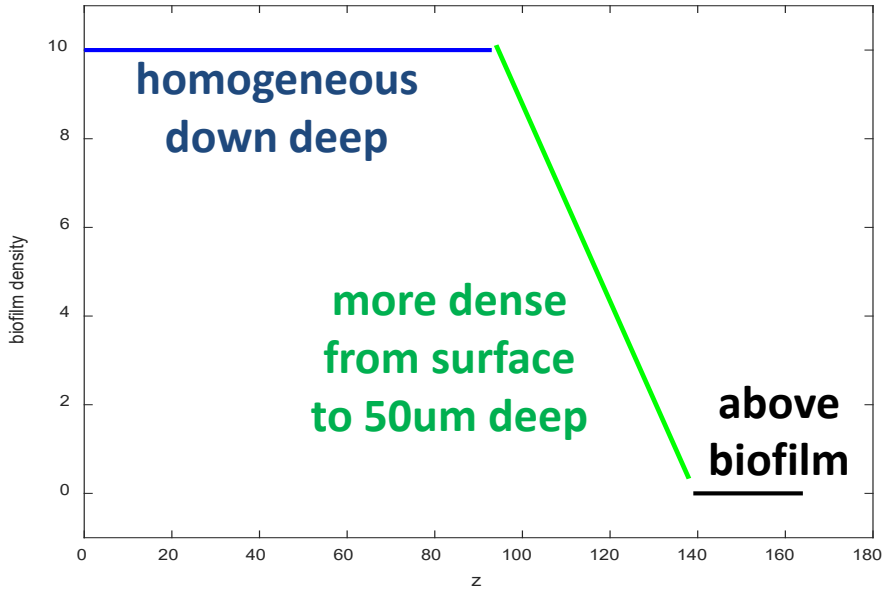
Model attenuation in each column of the image

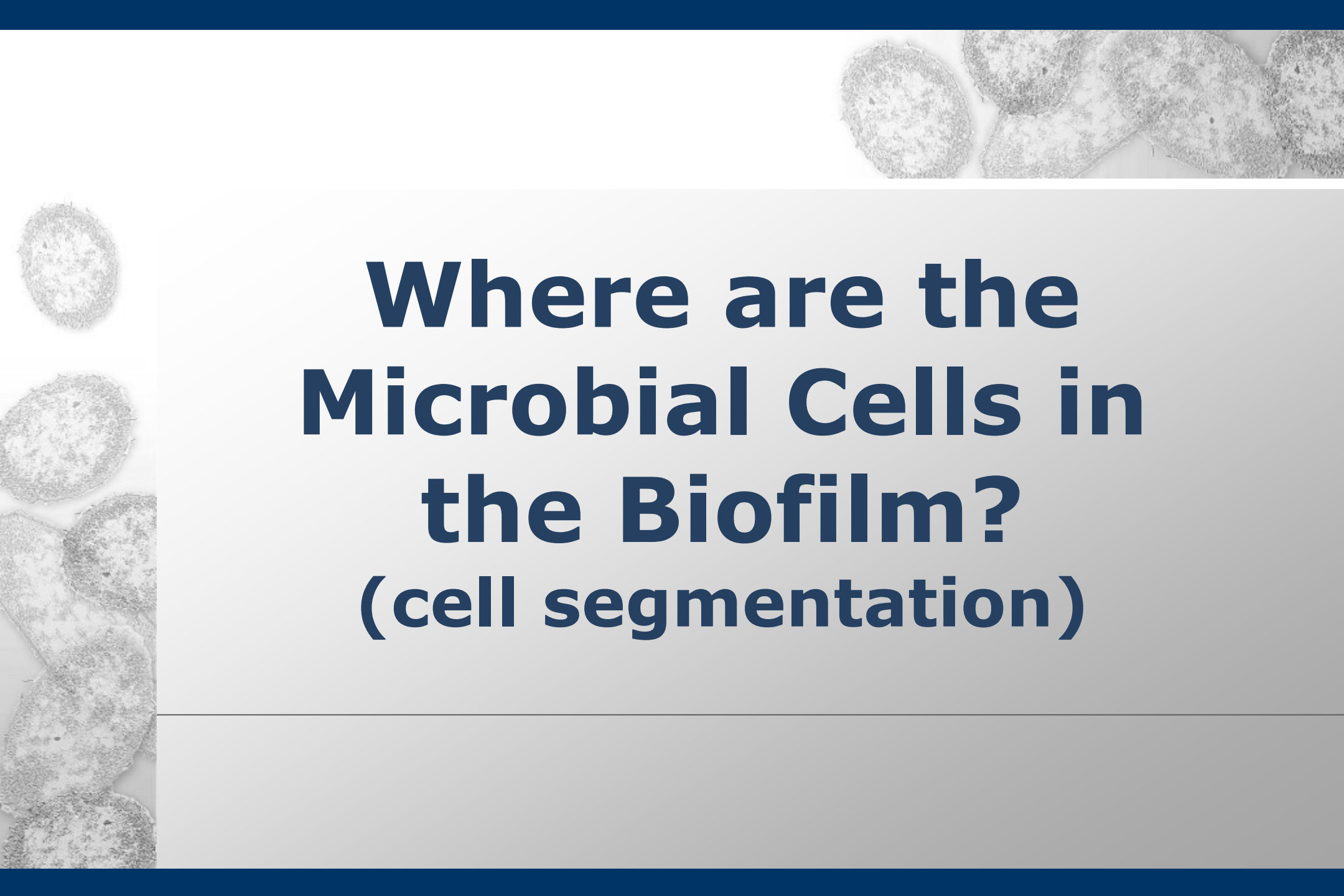
Different intensity profiles suggest different density profiles





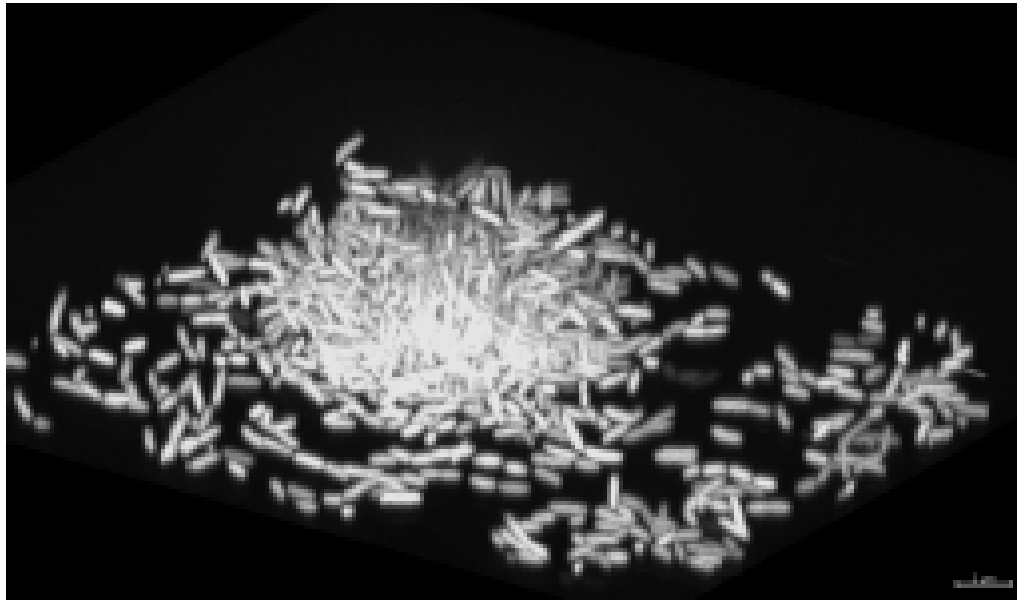




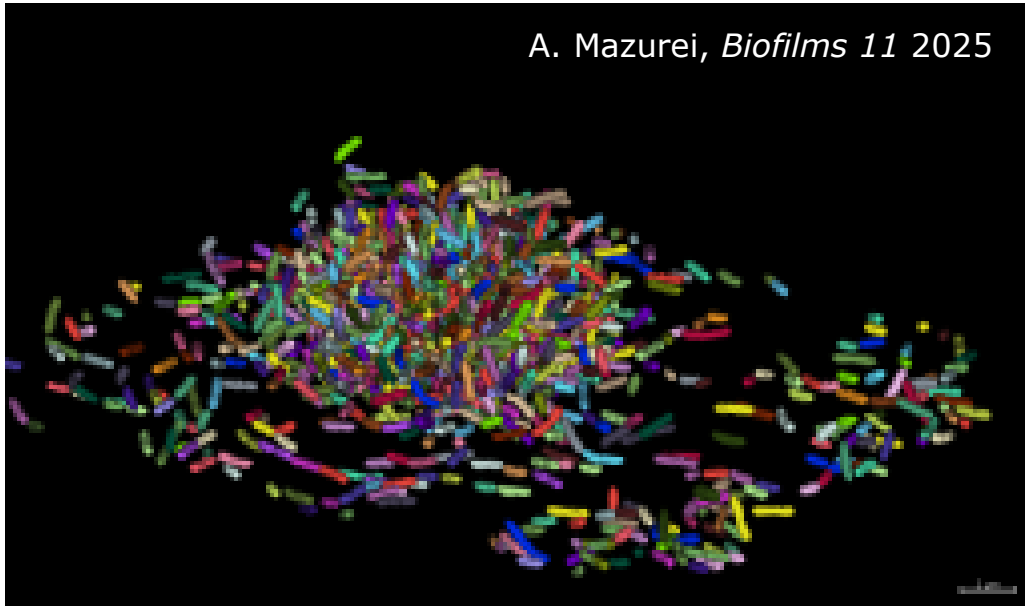


Where are the Microbial Cells in the Biofilm?
(cell segmentation)

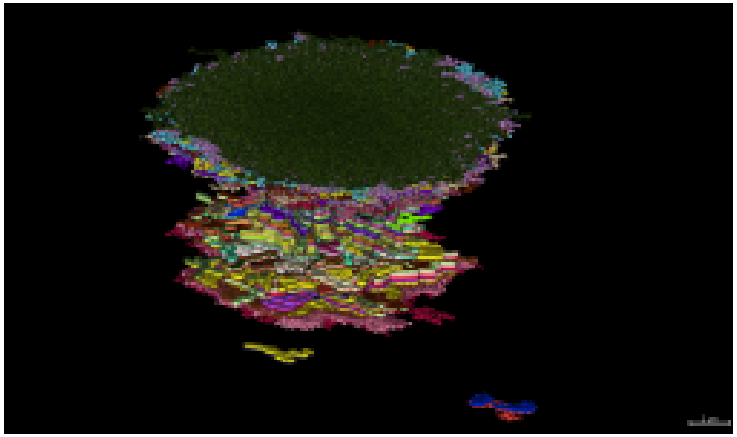
Cell segmentation: preparing to train



Raw gray scale image data

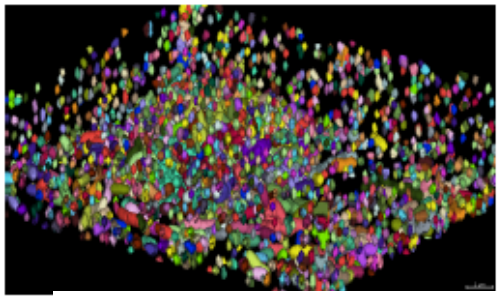


Human-annotated image data

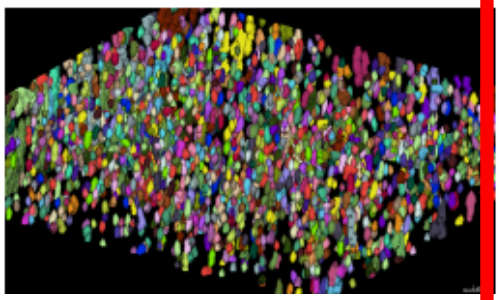


Segmentation by Thresholding Fails Miserably

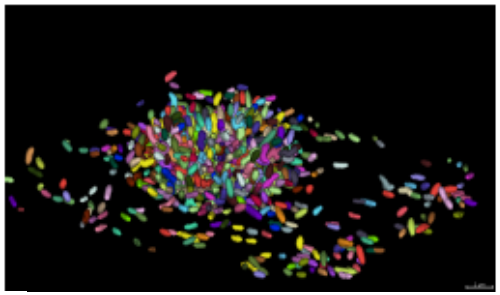
Four cell segmenters on 1 image: OTB v trained



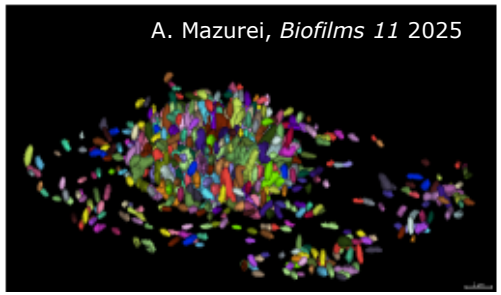
BCM3D-OTB



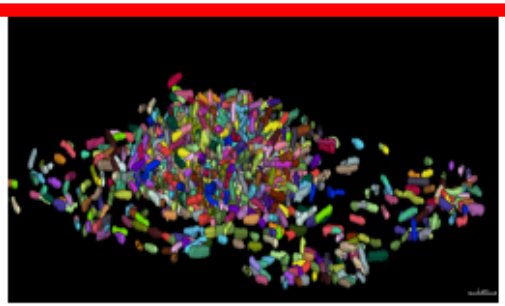
BCM3D -trained



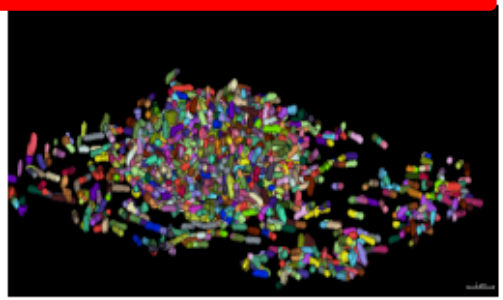
StarDist OPP-OTB



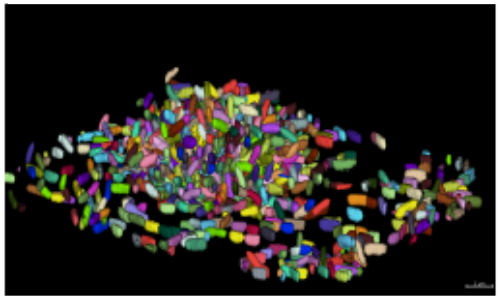
StarDist OPP -trained



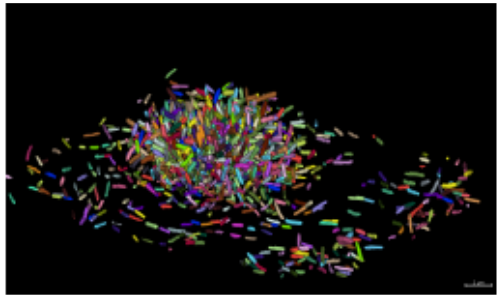
DeepSeeded-OTB



DeepSeeded -trained



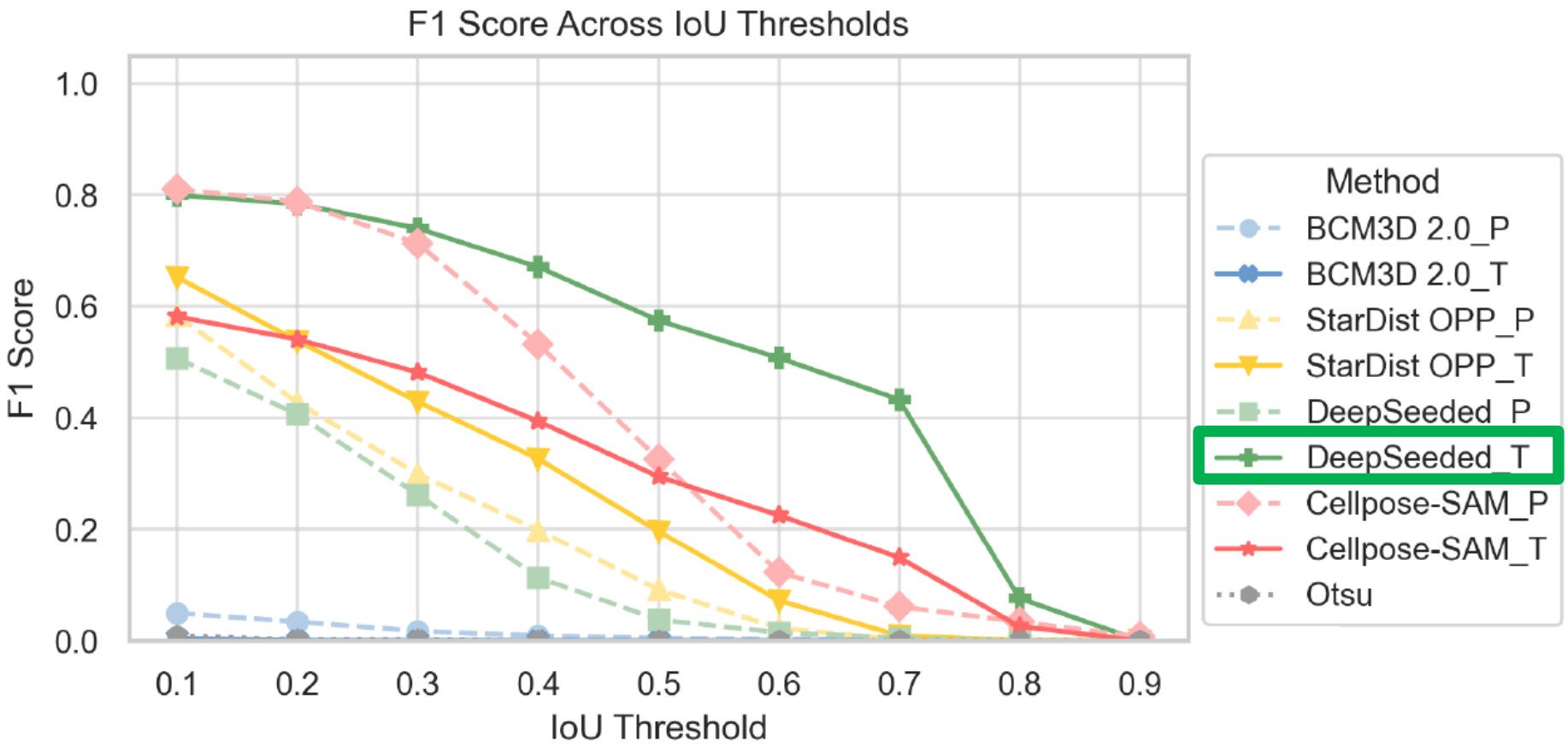
Cellpose-SAM-OTB



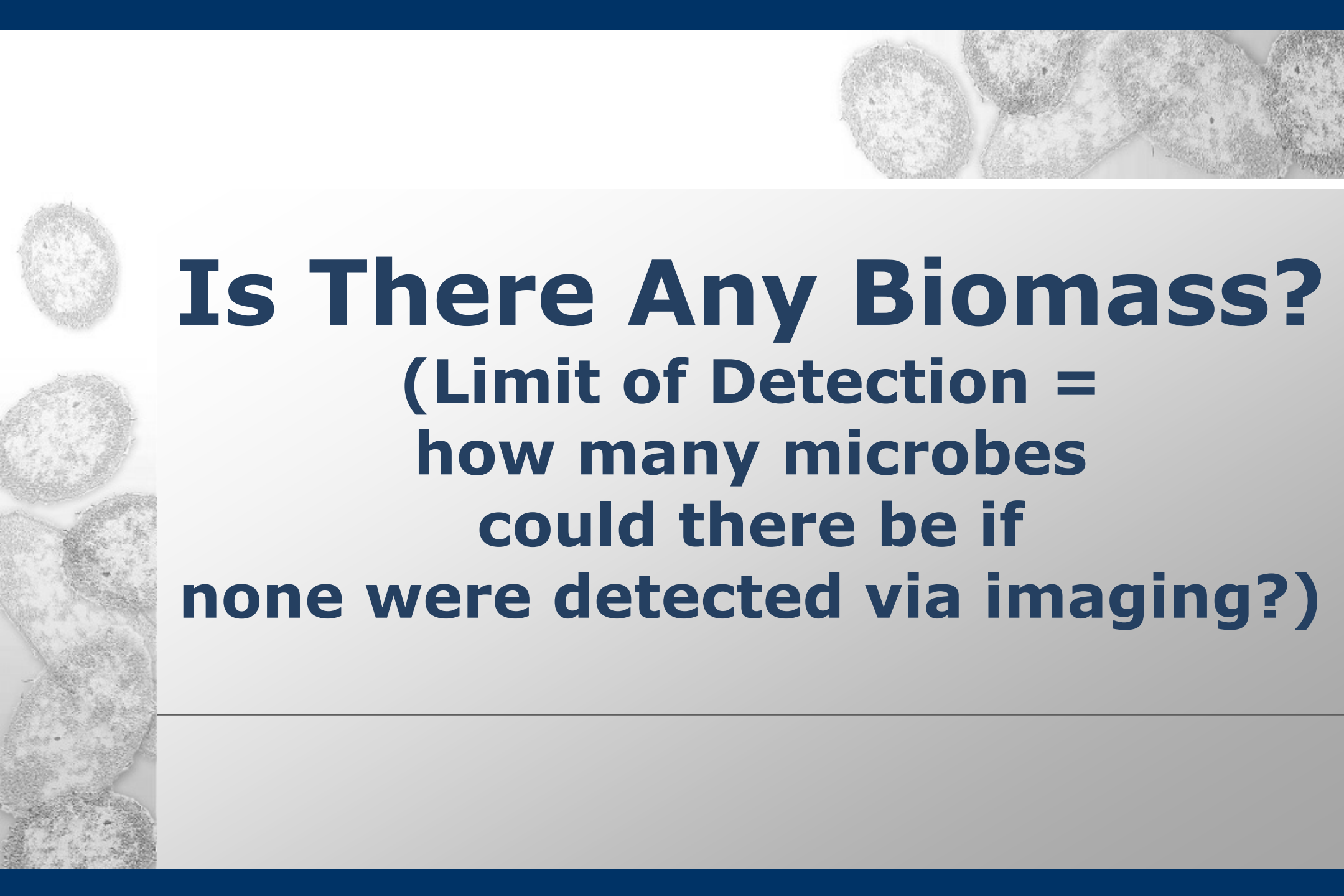
Cellpose-SAM -trained

Trained on 50 images

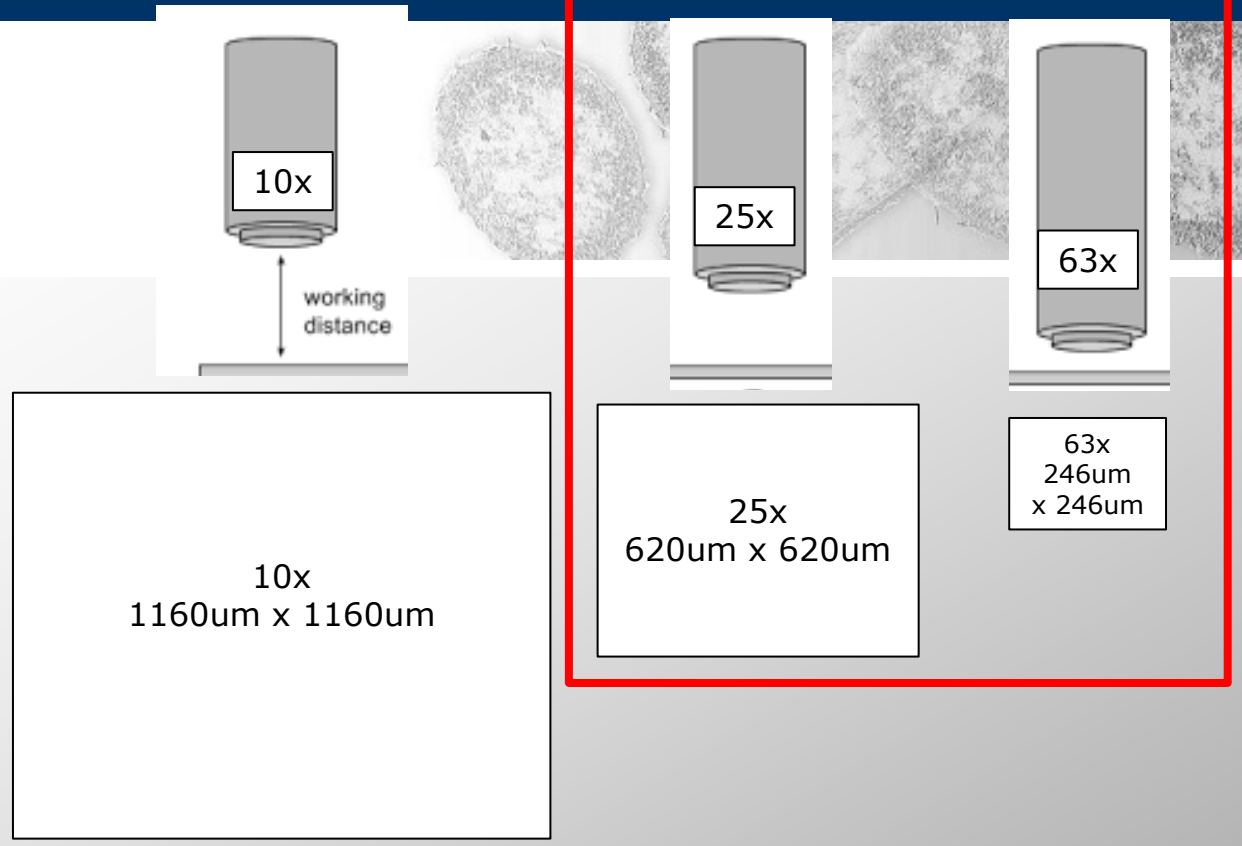
Four cell segmenters on : OTB v trained



Trained on 50 images
Tested on 21 images


Is There Any Biomass?
(Limit of Detection =
how many microbes
could there be if
none were detected via imaging?)

The **field of view (FOV)** changes with the objective

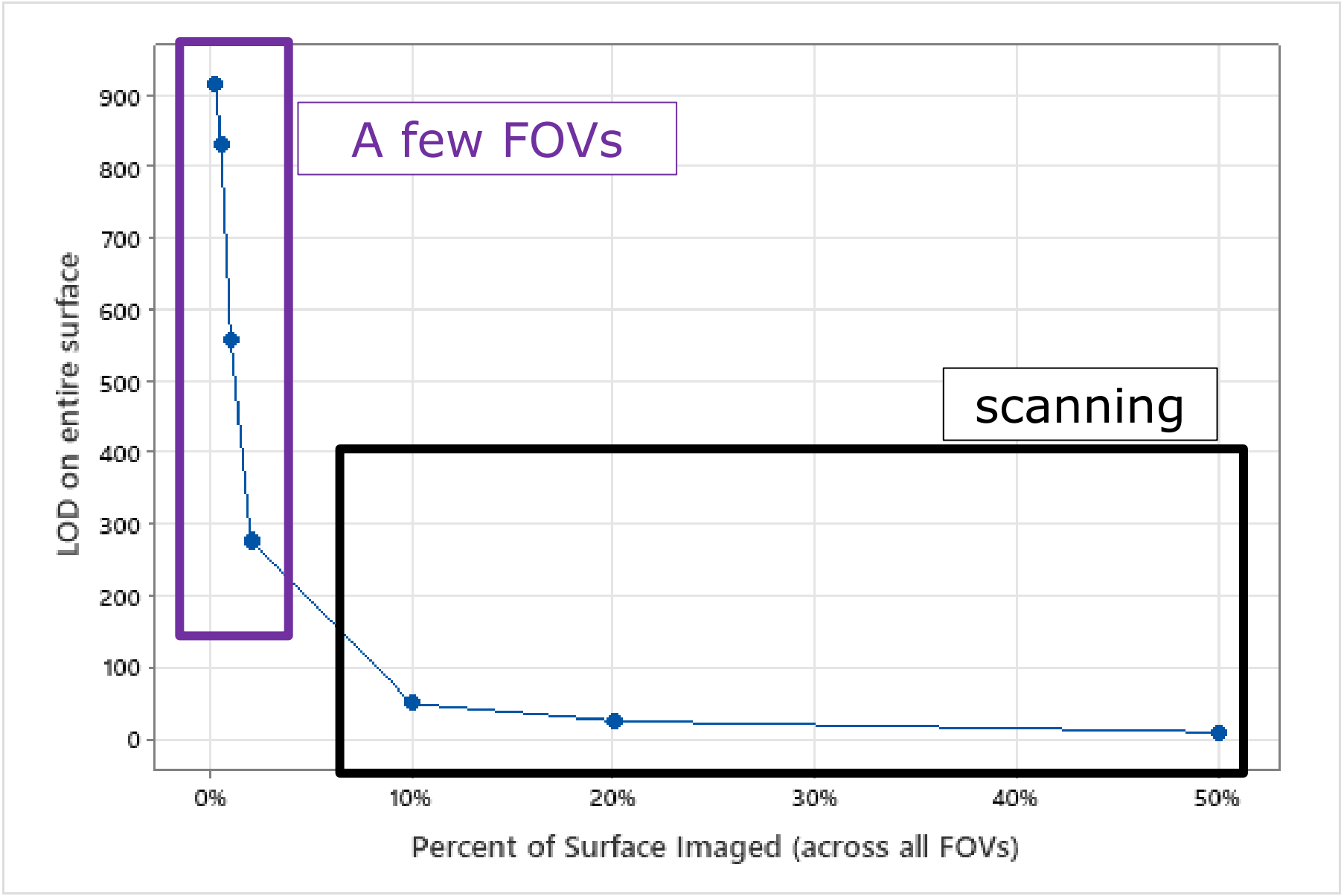


The **limit of detection (LOD)** for imaging:
For objectives that can reliably image individual microbes (e.g., 25x and higher), if no microbes (no biovolume) were found in 1 FOV imaged from 1 sample, then there's a 95% chance that there are up to

LOD = 5.6 CFU/FOV (as few as 3 CFU/FOV if Poisson)

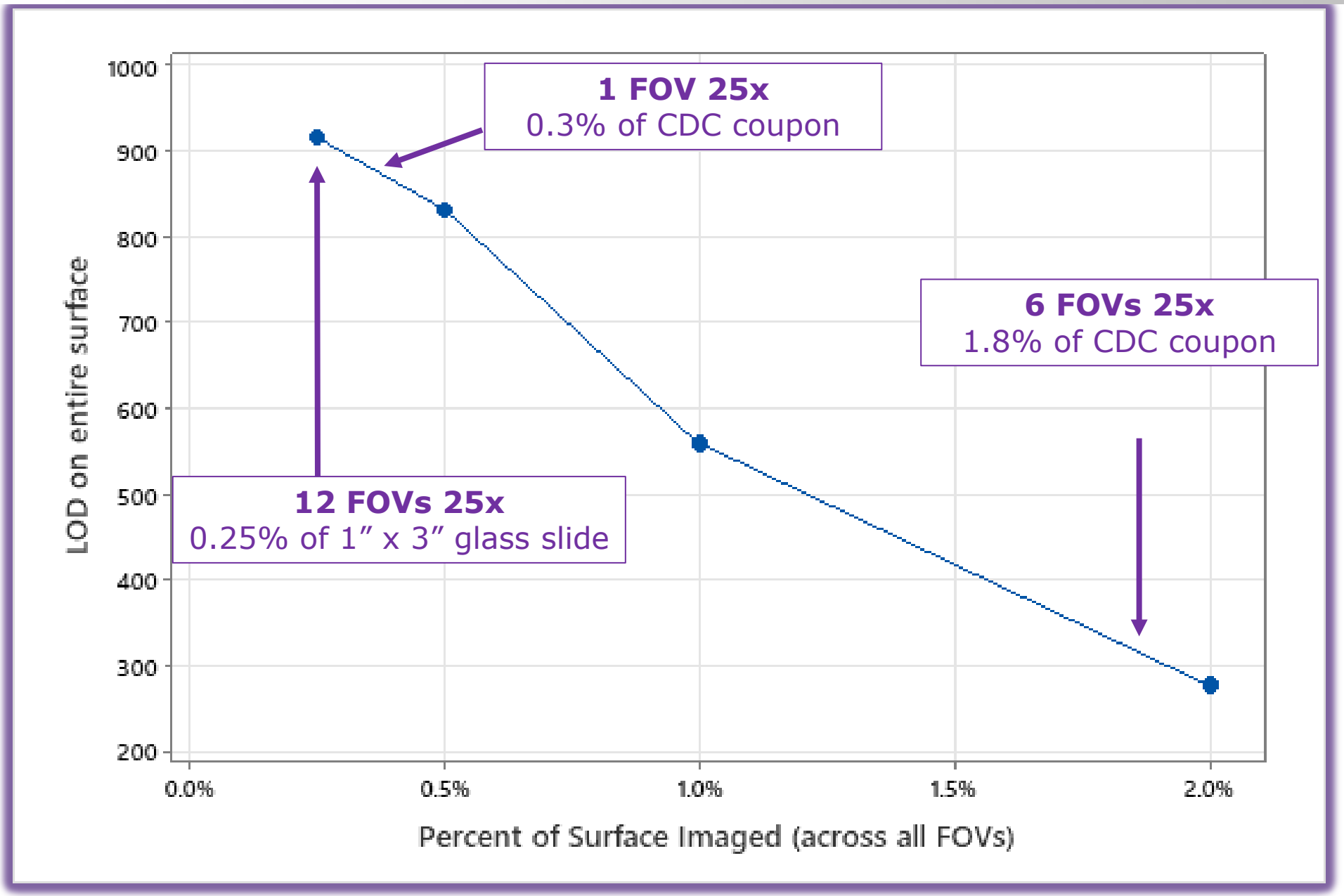
on average across the sample.

How many bugs when there are no bugs in 1 sample?

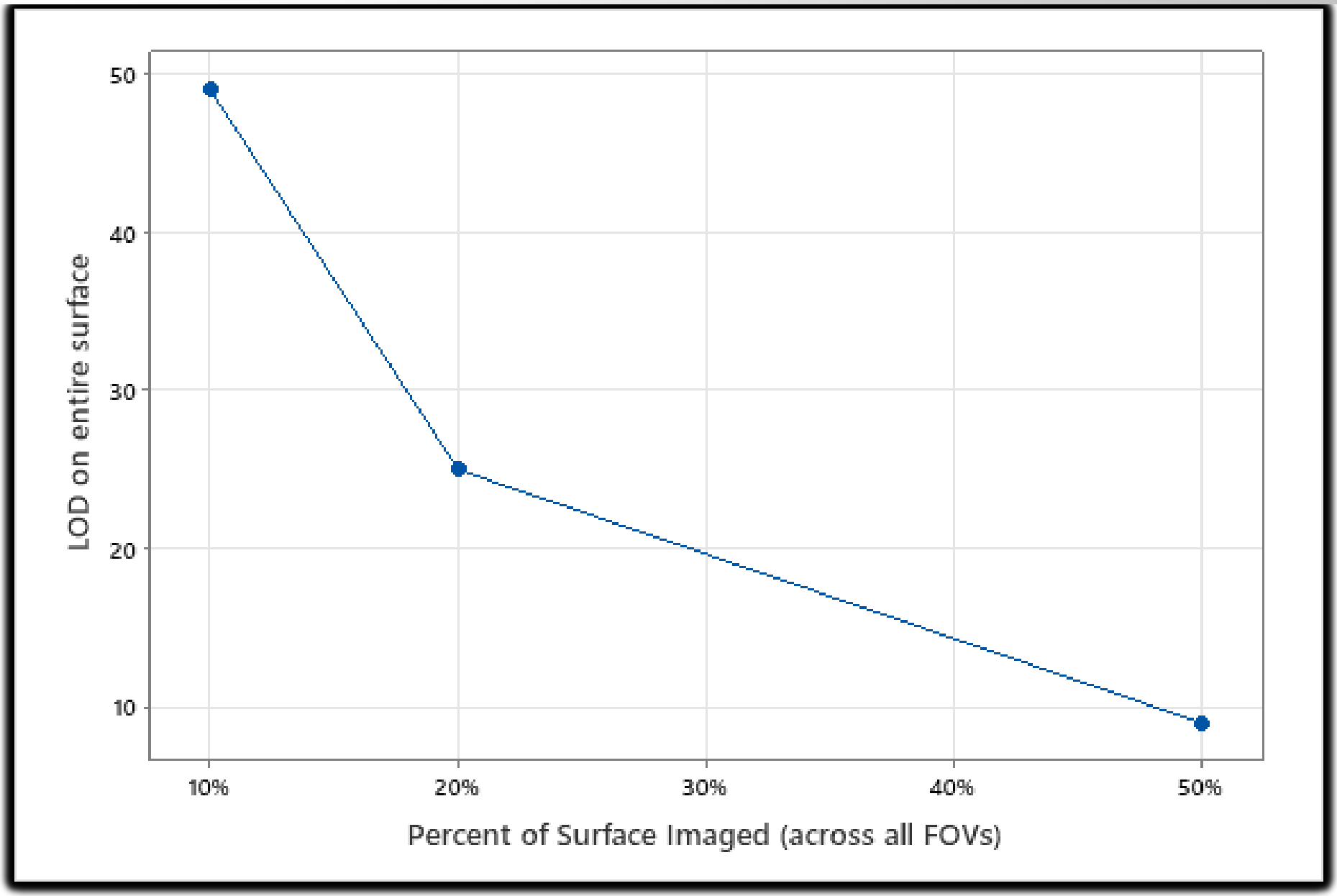


Christen & P, *J Ag Bio Env Statistics*, 2020

How many bugs when there are no bugs in a few FOVs?



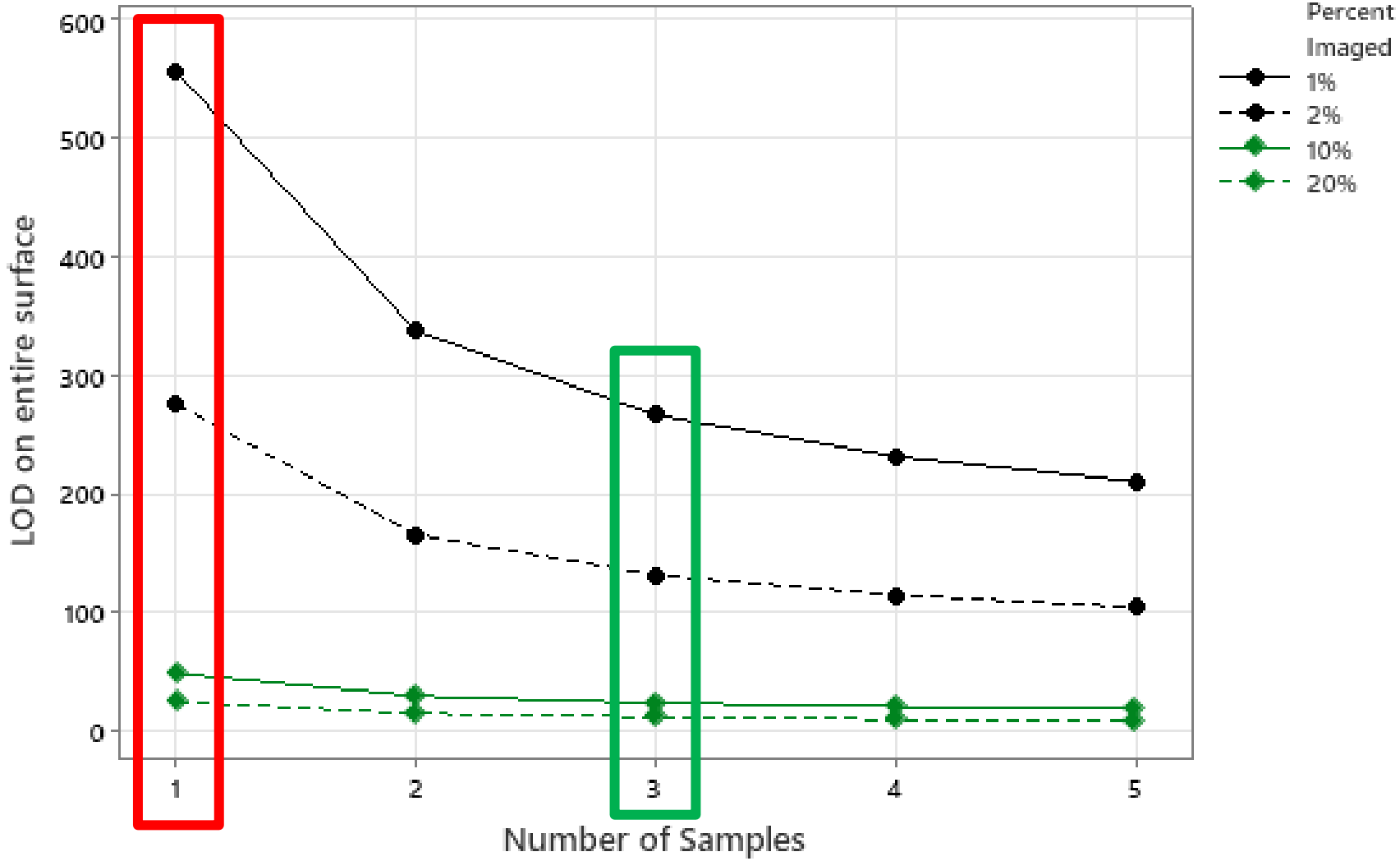
How many bugs when there are no bugs when “tile scanning”?



How many bugs as a function of the number of samples?

Examples in earlier slides were all for a single replicate sample

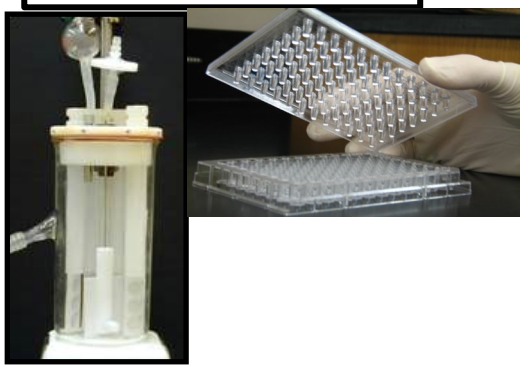
LOD when imaging three replicate samples.



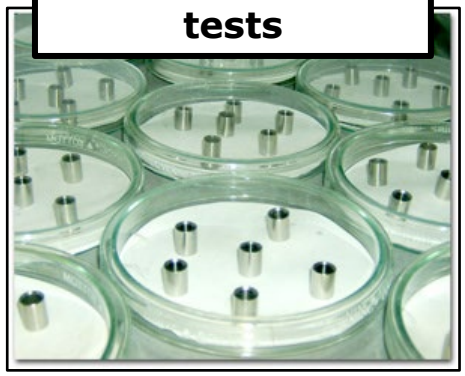
How Much Variability in Imaging Outcomes?

Variability of efficacy in CFUs is a function of efficacy

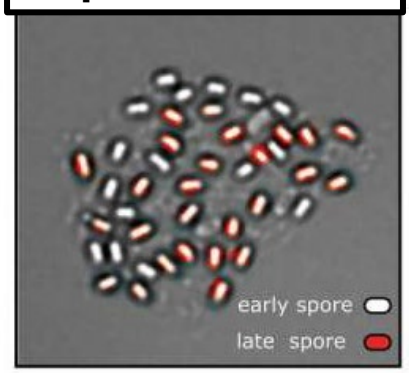
Biofilm tests

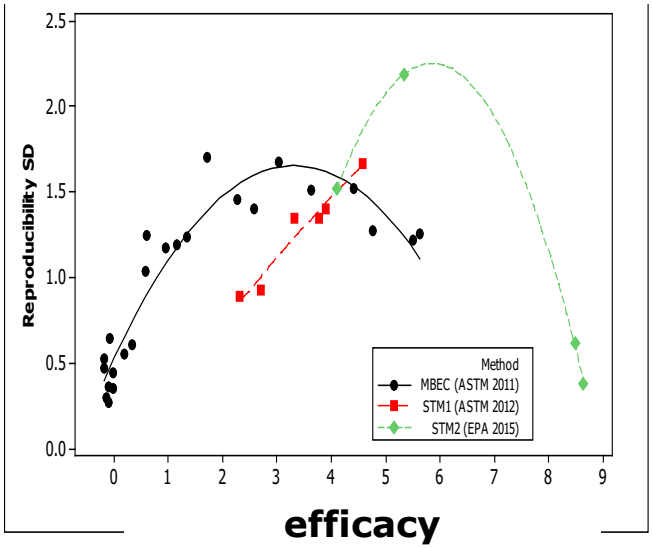


Dried surface tests

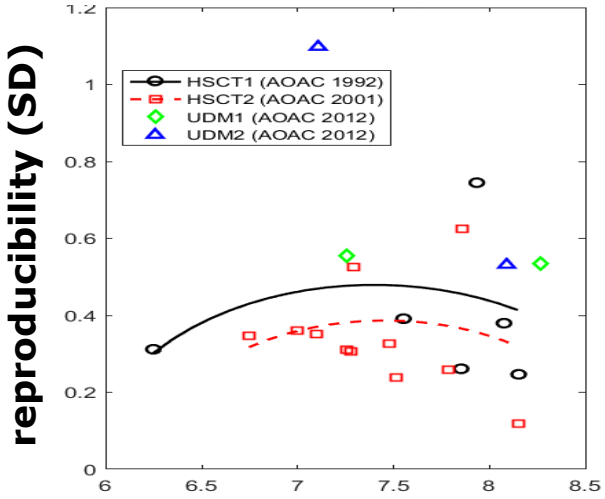


Sporicide tests

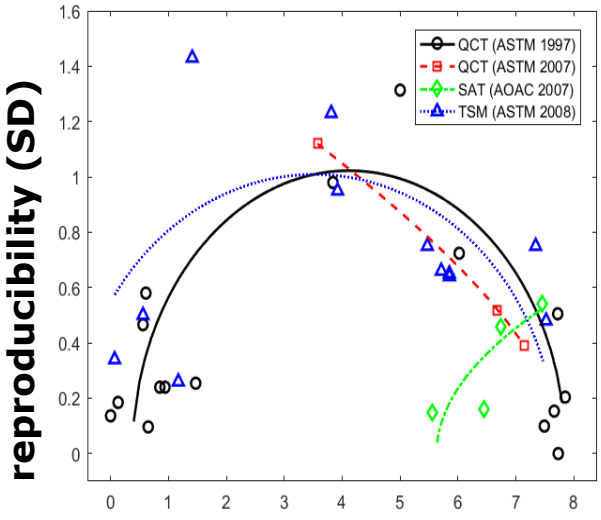




efficacy
(log reduction)

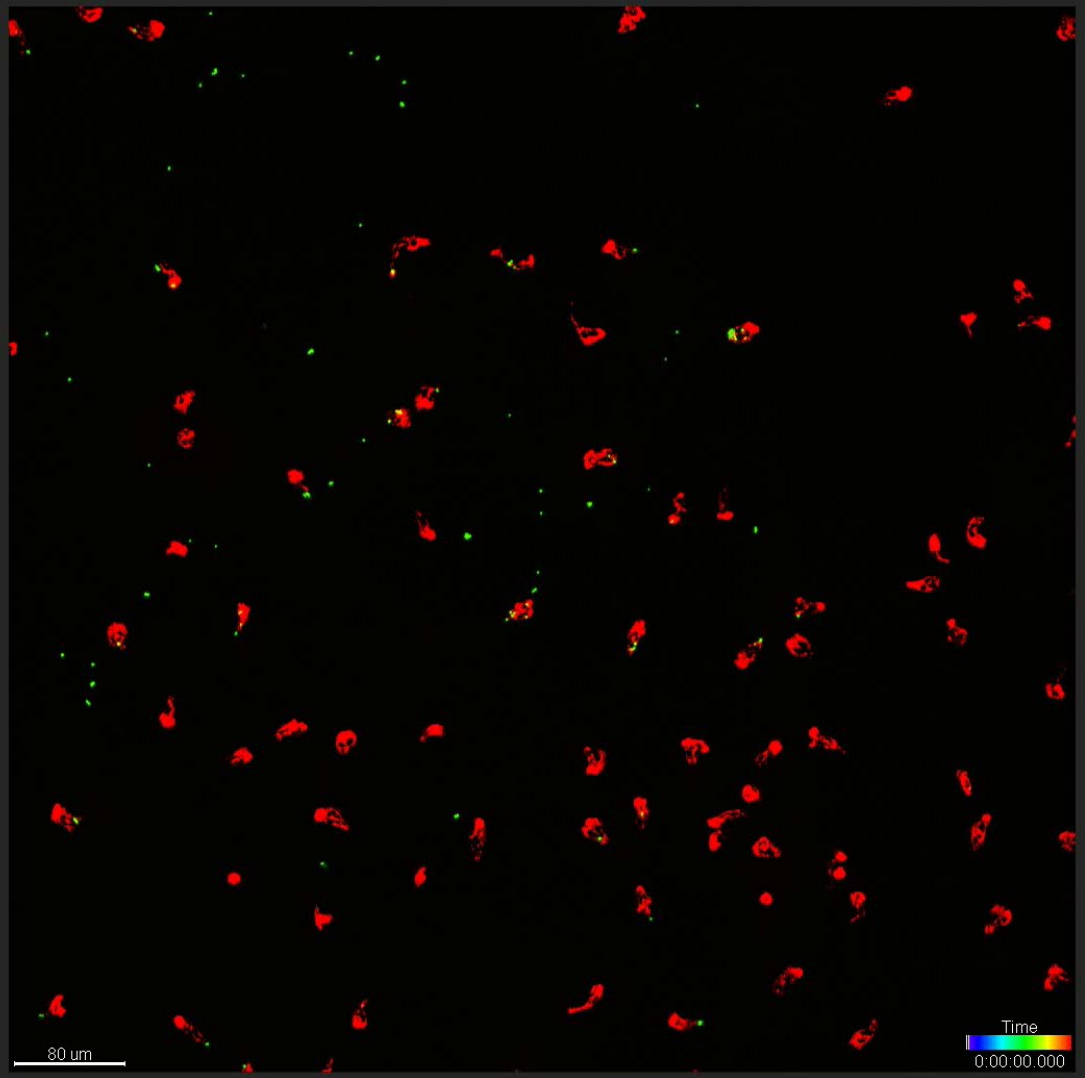


efficacy
(log reduction)

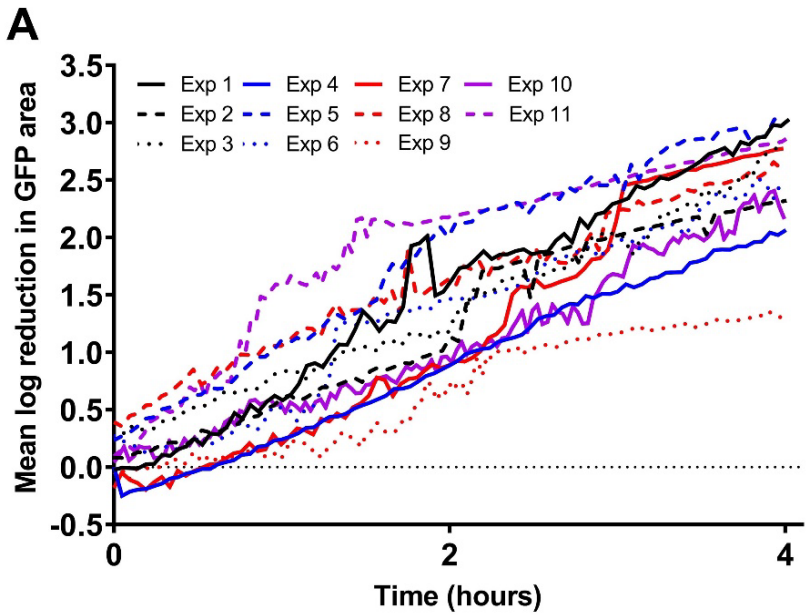


efficacy
(log reduction)

Neutrophil treatments applied to *Staph aureus*

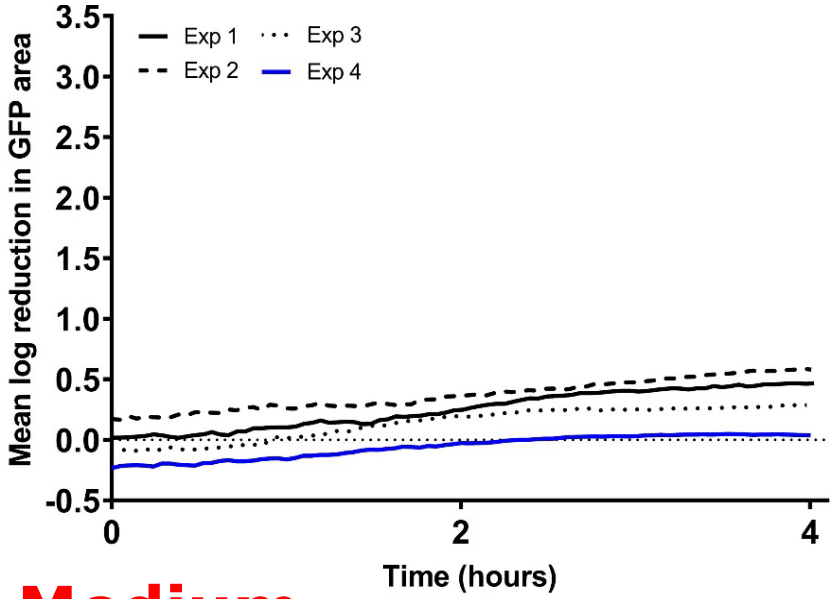


cultured overnight,
GFP Staph, red stained neutrophils
FOV: 620um x 620um x 20um
Pixilation: 512 x 512 x 20
1 image every 2-3 minutes over 4 hours

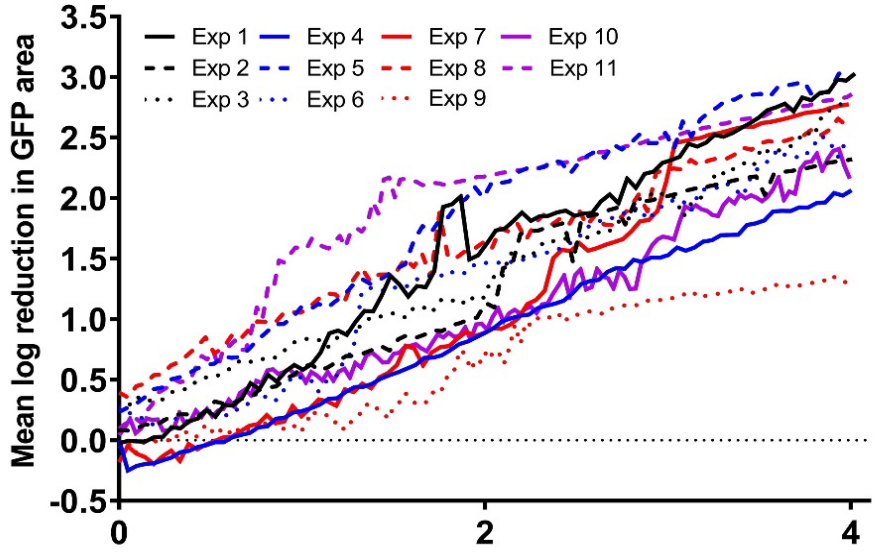


Pettygrove et al, *Biomaterials*, 2021

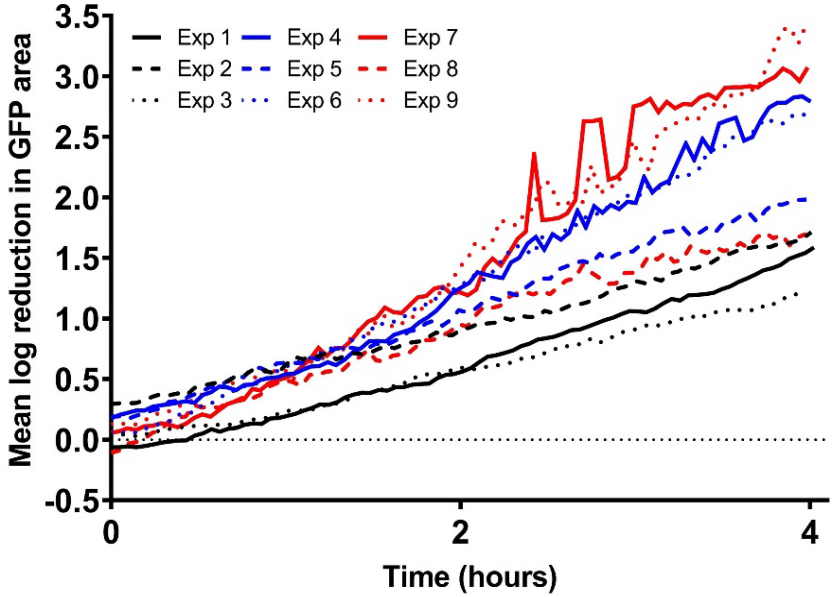
Low



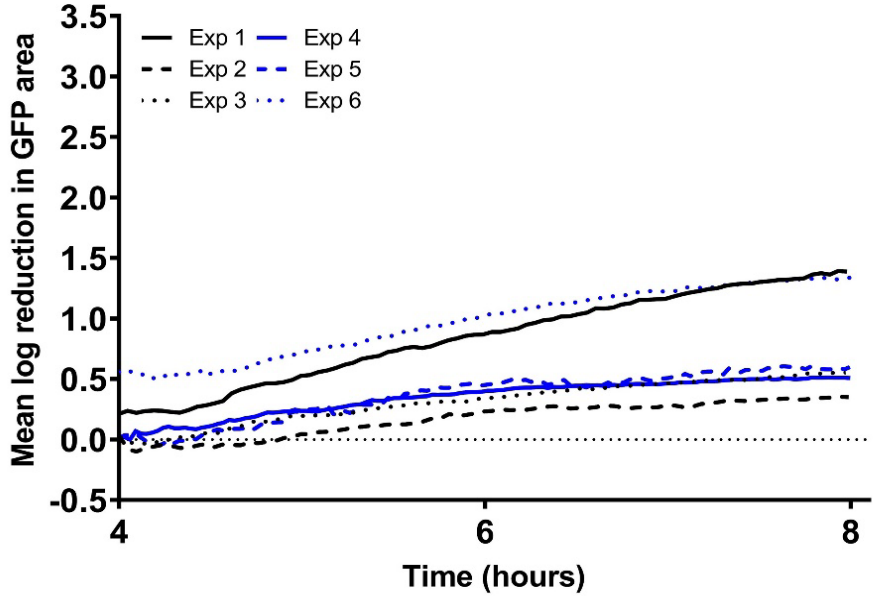
High applied early



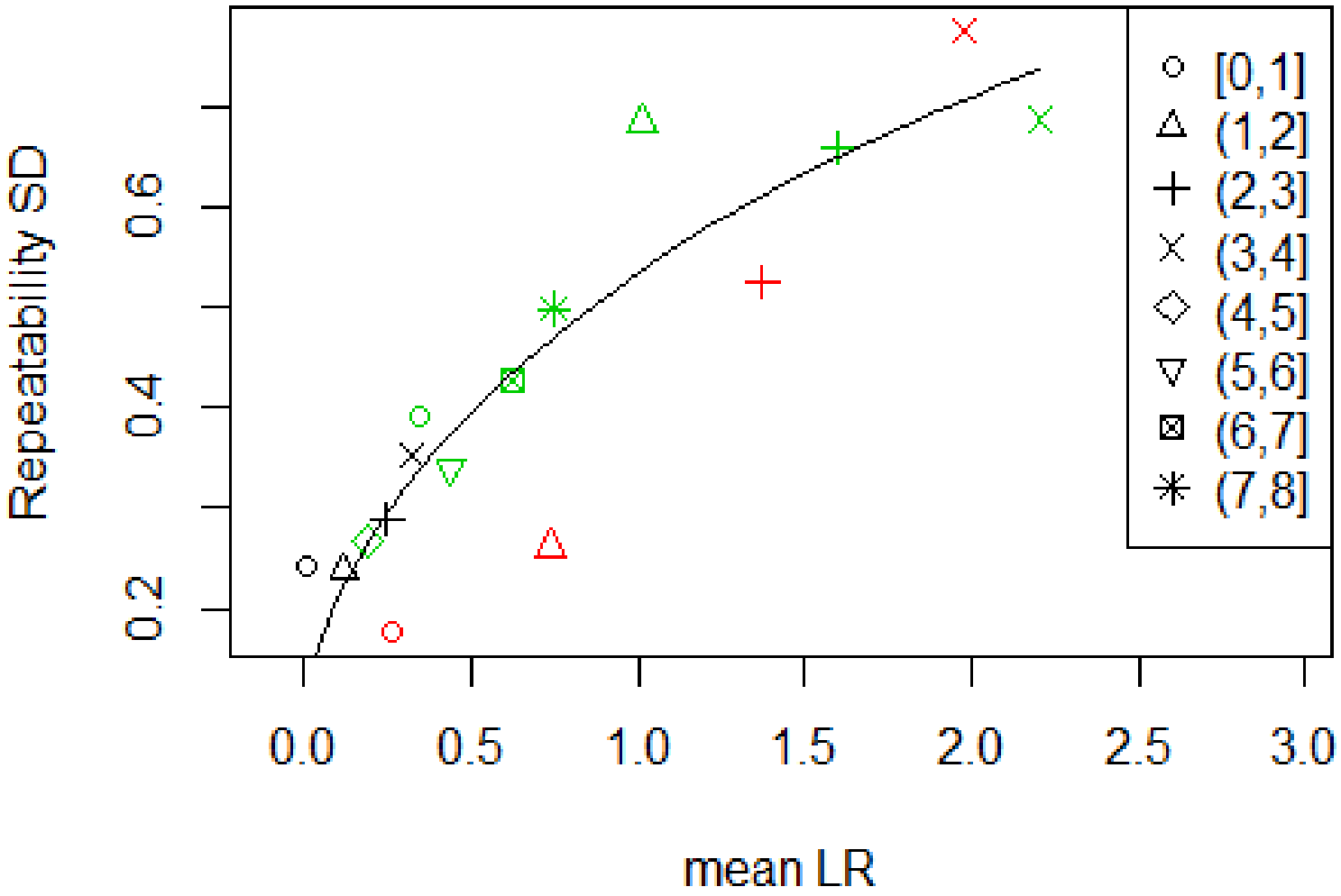
Medium



High applied late



Imaging variability is a function of efficacy



Black indicates LOW neutrophil treatment

Red indicates MEDIUM neutrophil treatment

Green indicates HIGH neutrophil treatment

How to perform calculations

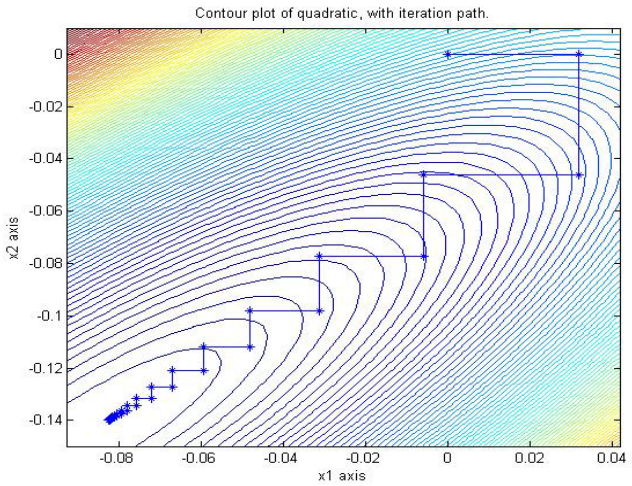
(variance assessment and experimental design)

computationally efficiently?

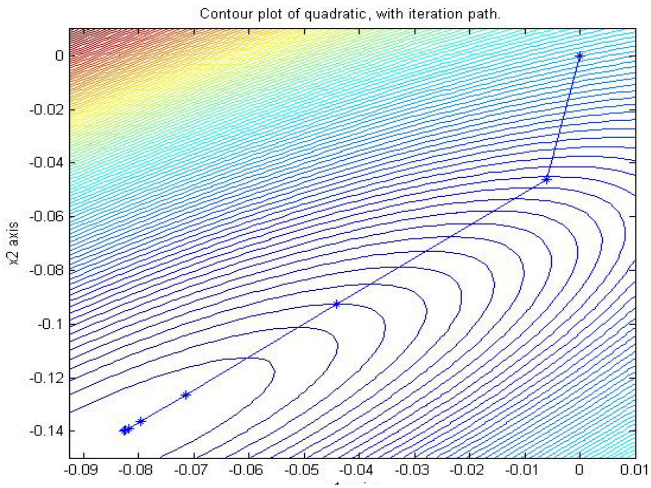
Polynomial iterative samplers to sample from large Gaussian $N(\Lambda^{-1}b, \Lambda^{-1})$

Solving $\Lambda x = b$:

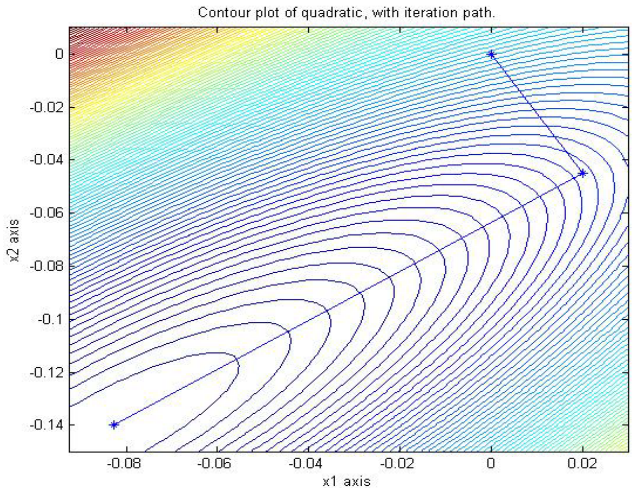
Fox & P, *SISC*, 2014
Fox & P, *Bernoulli*, 2017



Gauss-Seidel

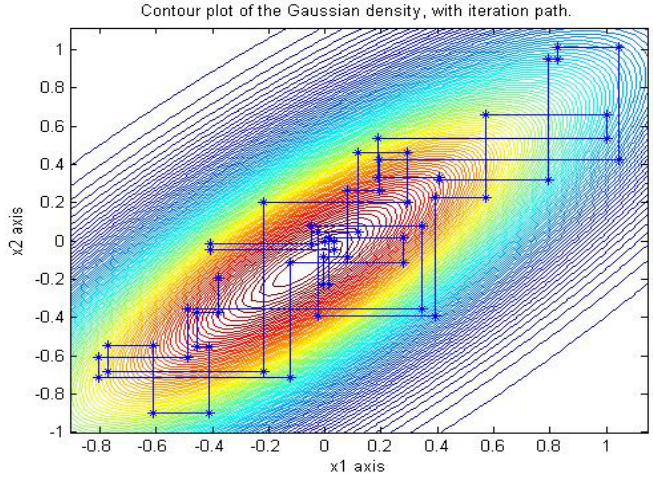


Chebyshev-Gauss-Seidel

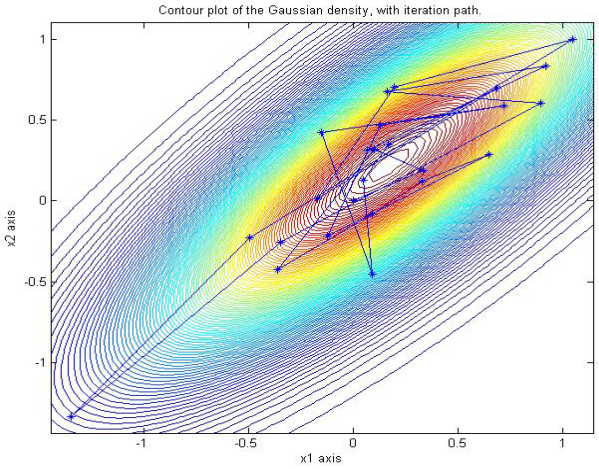


CG

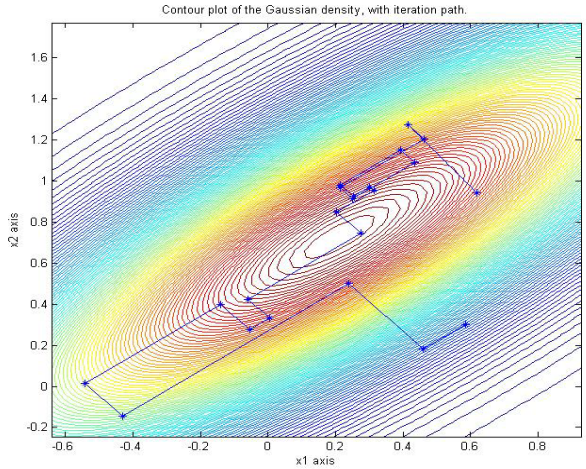
Sampling $y \sim N(0, \Lambda^{-1})$:



Gibbs



Chebyshev-Gibbs

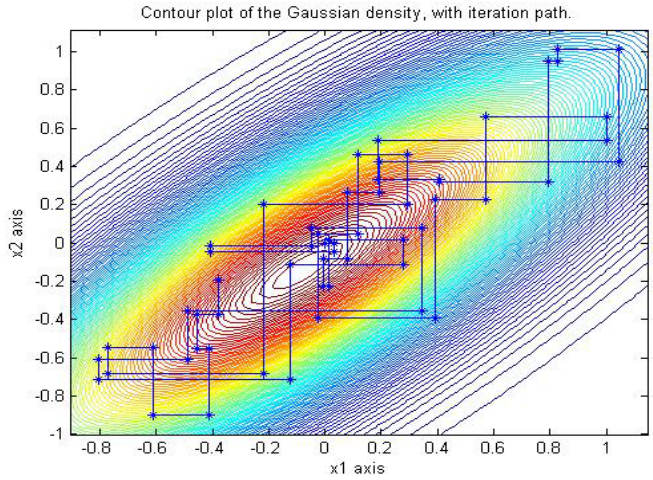


CG-Lanczos sampler

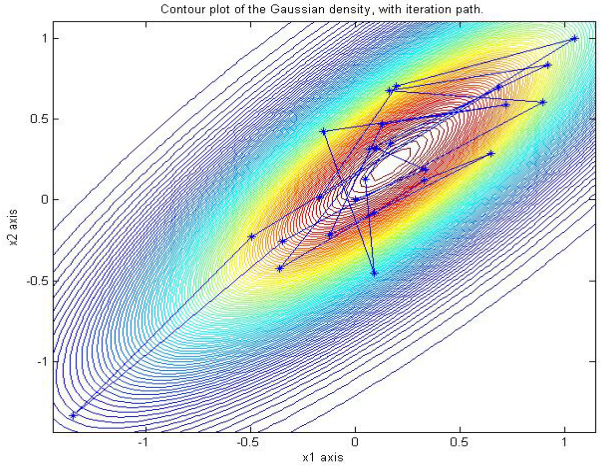
The sampler error decreases according to a polynomial,

$$(E(y^k) - \mu) = P_k(I-G)\,(E(y^0) - \mu)$$
$$(\Lambda^{-1} - \mathrm{Var}(y^k)) = P_k(I-G)\,(\Lambda^{-1} - \mathrm{Var}(y^0))\,P_k(I-G)^T$$

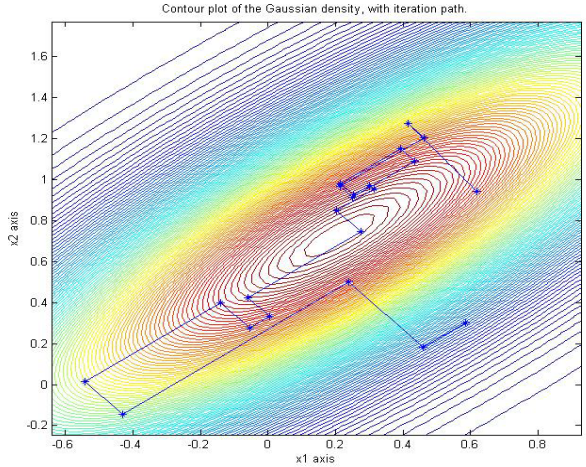
$$(\Lambda^{-1} - \mathrm{Var}(y^k))v = 0$$
for any Krylov vector v



Gibbs



Chebyshev-Gibbs



CG-Lanczos

$P_k(I-G) = G^k$,

with error reduction factor

$p(G)^2$

$P_k(I-G)$

k^{th} order Chebyshev polyomial,

optimal variance asymptotic average reduction factor is

$$\sigma^2 = \left(\frac{1-\sqrt{cond(I-G)}}{1+\sqrt{cond(I-G)}}\right)^2$$

$\mathrm{Var}(y^k)$ is the k^{th} order CG polynomial

converges in a finite number of steps* in a Krylov space depending on eig(I-G)

In $R^{512 \times 512 \times 512 \times 512}$, polynomial iterative sampling is 1/10,000th of the cost of conventional Cholesky sampling

Each Gaussian sample of a single 512 x 512 movie frame costs:

6.9×10^{10} flops for a Cholesky matrix factorization
[$O(b^2n) = O(512^4)$]

9.7×10^7 flops for an iterative Chebyshev sampler
[$O(\#\text{iters} \times 2n) = O(185 \times 2 \times 512^2)$]

2.2×10^7 flops for an iterative CG sampler
[$O(\#\text{iters} \times n) = O(85 \times 512^2)$]

We need a Biofilm Image Library!

An open-access, community-driven repository of images, meta data and computationally efficient models to help address challenges when analyzing images of biofilms:

- **Is there any biofilm?** many FOVs and many samples are needed if a low limit of detection is desired.
- **How much biofilm?** Calculating even a simple biovolume of a thick biofilm can be challenging for some software (Imaris).
- **Where are the cells in the biofilm?** An open area of research.
- **Which species are present in the biofilm?** An open area of research.
- **How do multiple species correlate in space?** Pixel based measures exist, cell-based measures are … an open area of research.
- **Which morphologies indicate pathogenicity or susceptibility or?** an open area of research.

Questions?

www.biofilm.montana.edu