Bob Austin
Dept. of Physics
Princeton University

Falling Uphill: Acceleration of the Emergence of Bacterial Antibiotic Resistance

OR:

"A-tale-of-an-ingenious-experiment-with-a-dubious-interpretation"

(from web site devoted to attacking me)

J. B. S. Haldane: (yes, that Haldane, not Eddington or Duncan Haldane's unknown great-Uncle):

"I have no doubt that in reality the future will be vastly more surprising than anything I can imagine. Now my own suspicion is that the Universe is not only *queerer* than we suppose, but *queerer* than we **can** suppose."

"The star has to go on radiating and radiating and contracting and contracting until, I suppose, it gets down to a few km. radius, when gravity becomes strong enough to hold in the radiation, and the star can at last find peace. ... I think there should be a law of Nature to prevent a star from behaving in this absurd way!" Eddington

Good thing it was Haldane and not Eddington who actually said this, for Eddington proved to not believe the Universe was *queerer* than he could suppose.

Experiments!!!

Qiucen ("John") Zhang, Guillaume Lambert, David Liao, Guillaume Lambert, Grad Students, Princeton University Physics

Dr. Kristelle Robin, Institute for Advanced Studies, Hong Kong University of Science and Technology

In collaboration with:

Prof. Terry Hwa and Rutger Hermsen, UCSD Physics

Prof. Nader Pourmand, John Kim: UC Santa Cruz Sequencing Center

I. WHY am I doing this:

A) The Physics of Cancer (why I am not pure evil)

B) Fundamental Questions in Evolution Dynamics.

II. The Basic Experiment: Resistance in 10 hrs.

III. Is It Really De-Novo That Quickly?

IV. Genetics.

V. Toy Story

I. WHY am I doing this:

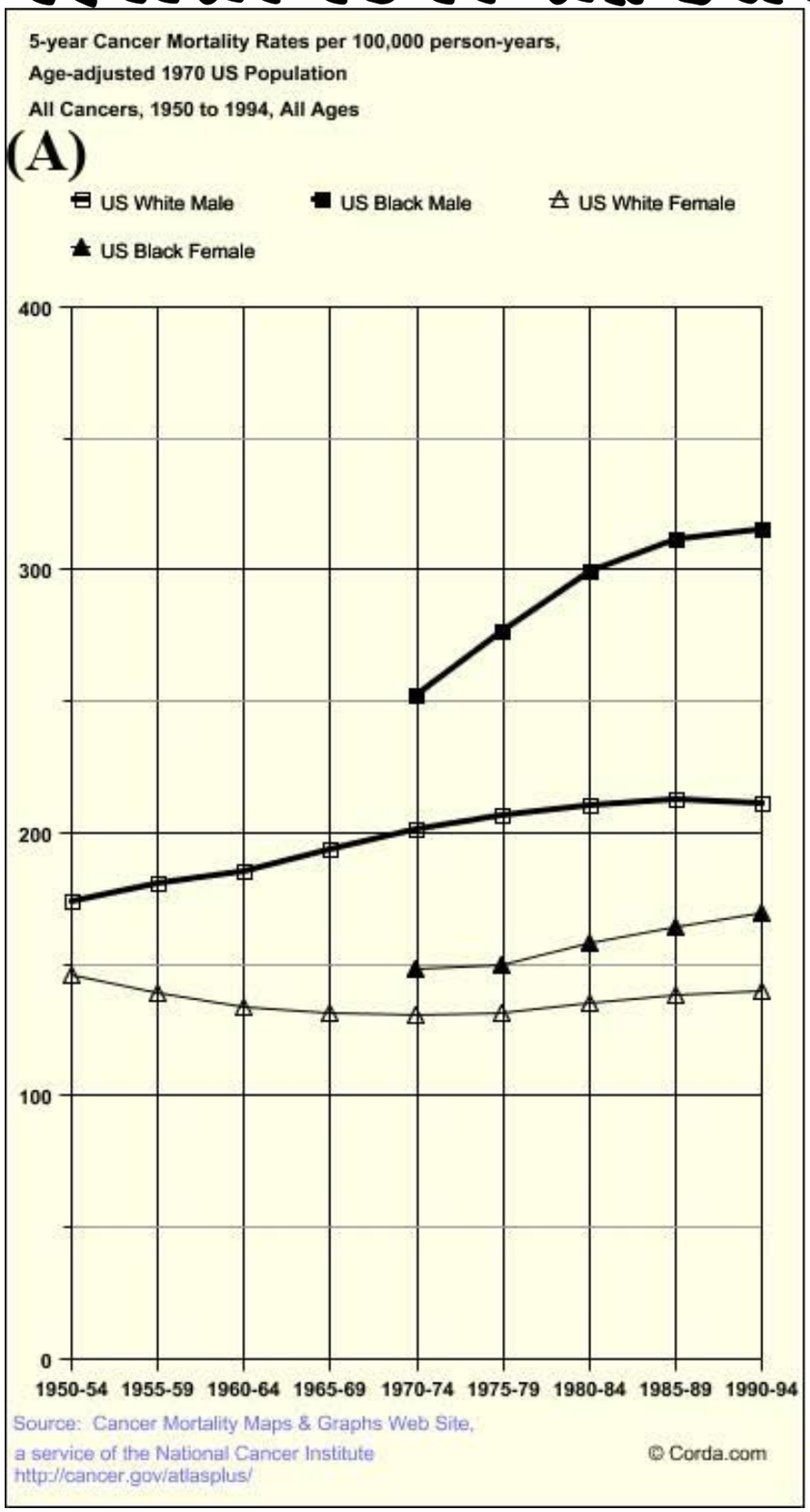
A) The Physics of Cancer (why I am not pure evil).

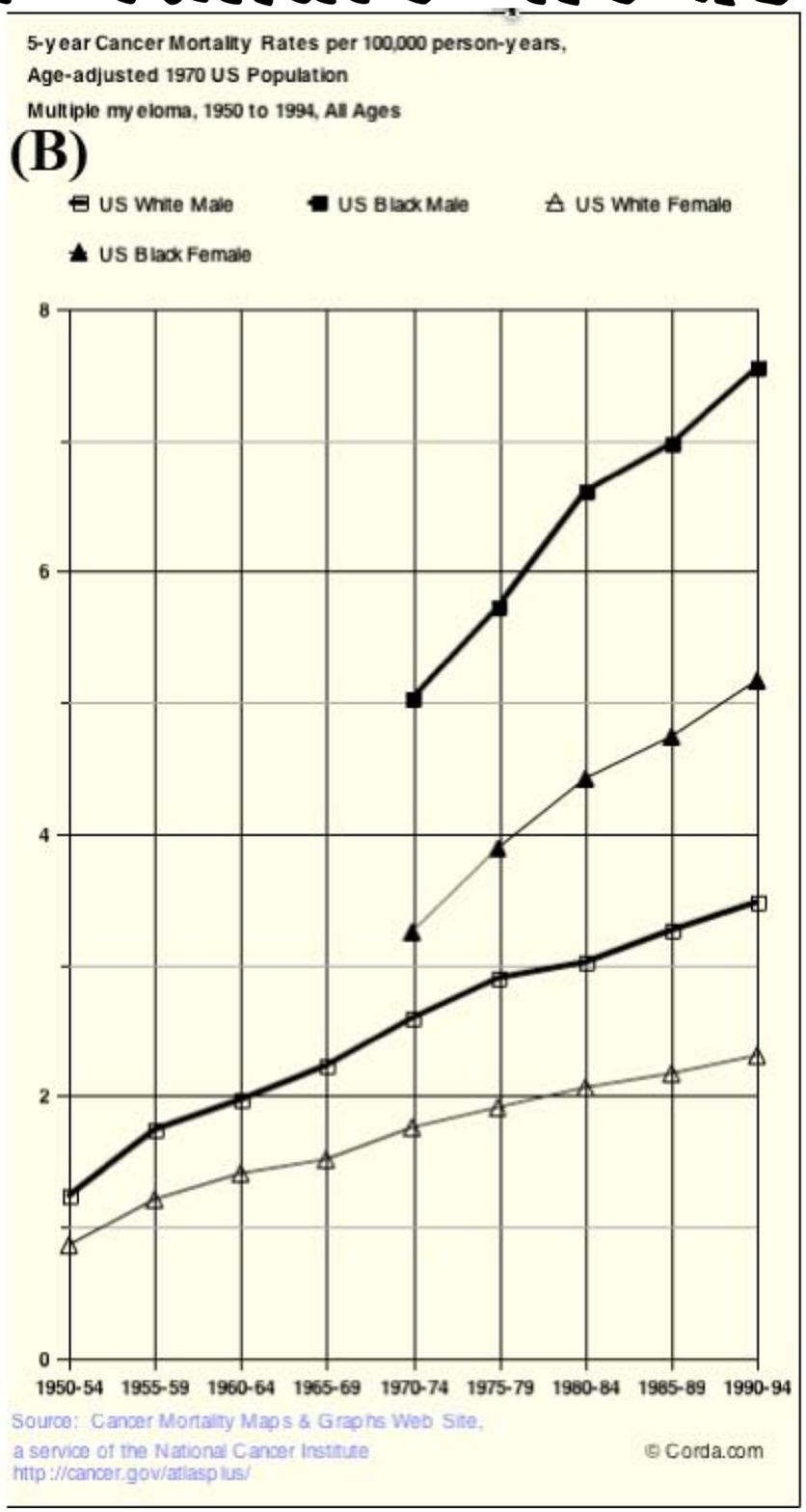
Usually I get stoned, when I suggest that maybe classical Darwinian theory is incomplete and somewhat misleading, and rightly so I would add.

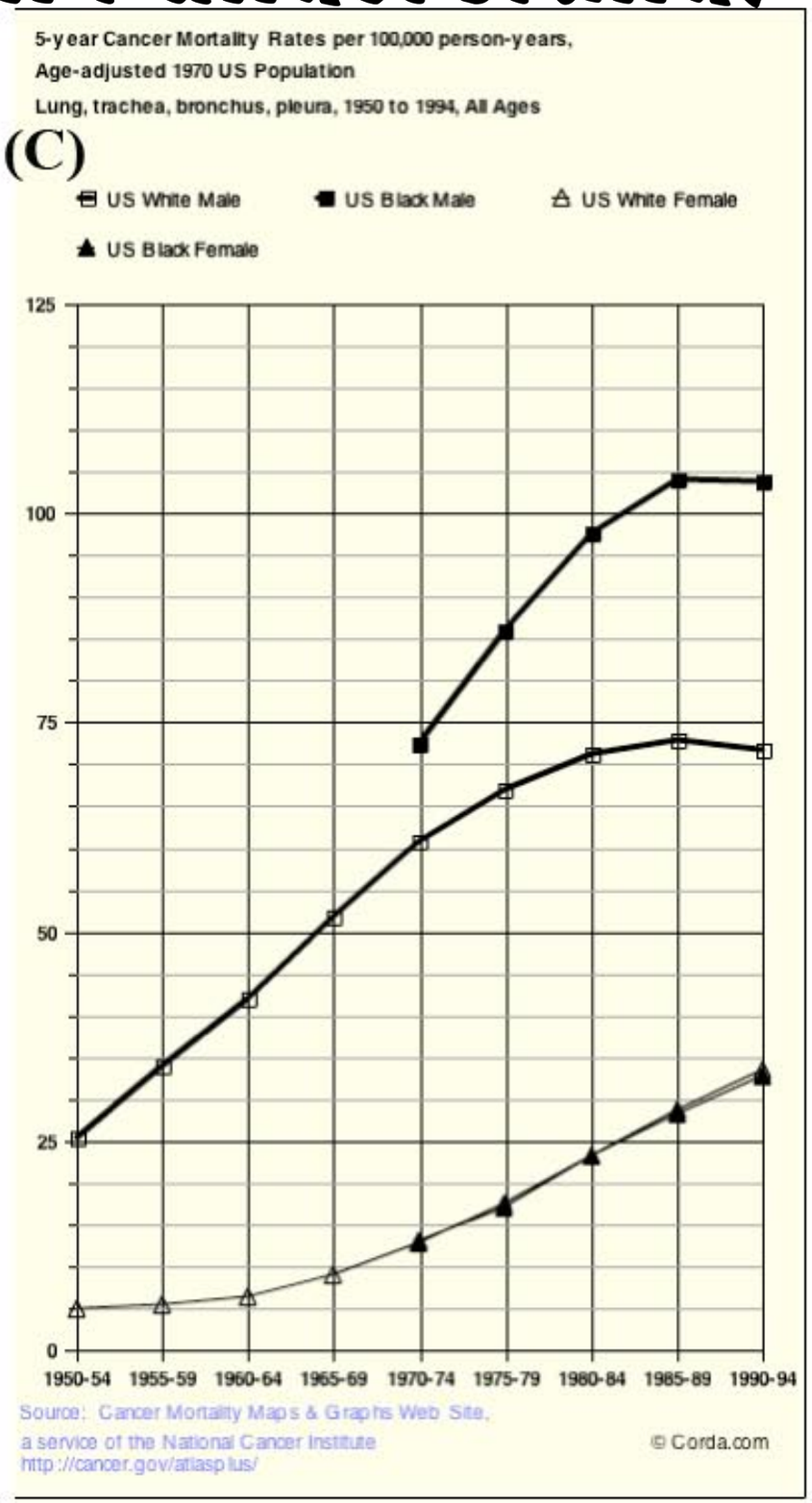
“I could say something trite about the hazards of letting physicists do biology. But the point is that Austin's experiments are ingenious, beautiful, and useful. Unfortunately, he has gotten totally out of his depth in interpreting them and would benefit by bringing an experienced evolutionary biologist (along with an experienced cell biologist) into his team to help interpret the results. Until he does so, he will remain on the margins.”

One must be certified by the biologists that a complete brain erasure and re-program has been done before we can do biology!

What is it about "failure" we don't understand?

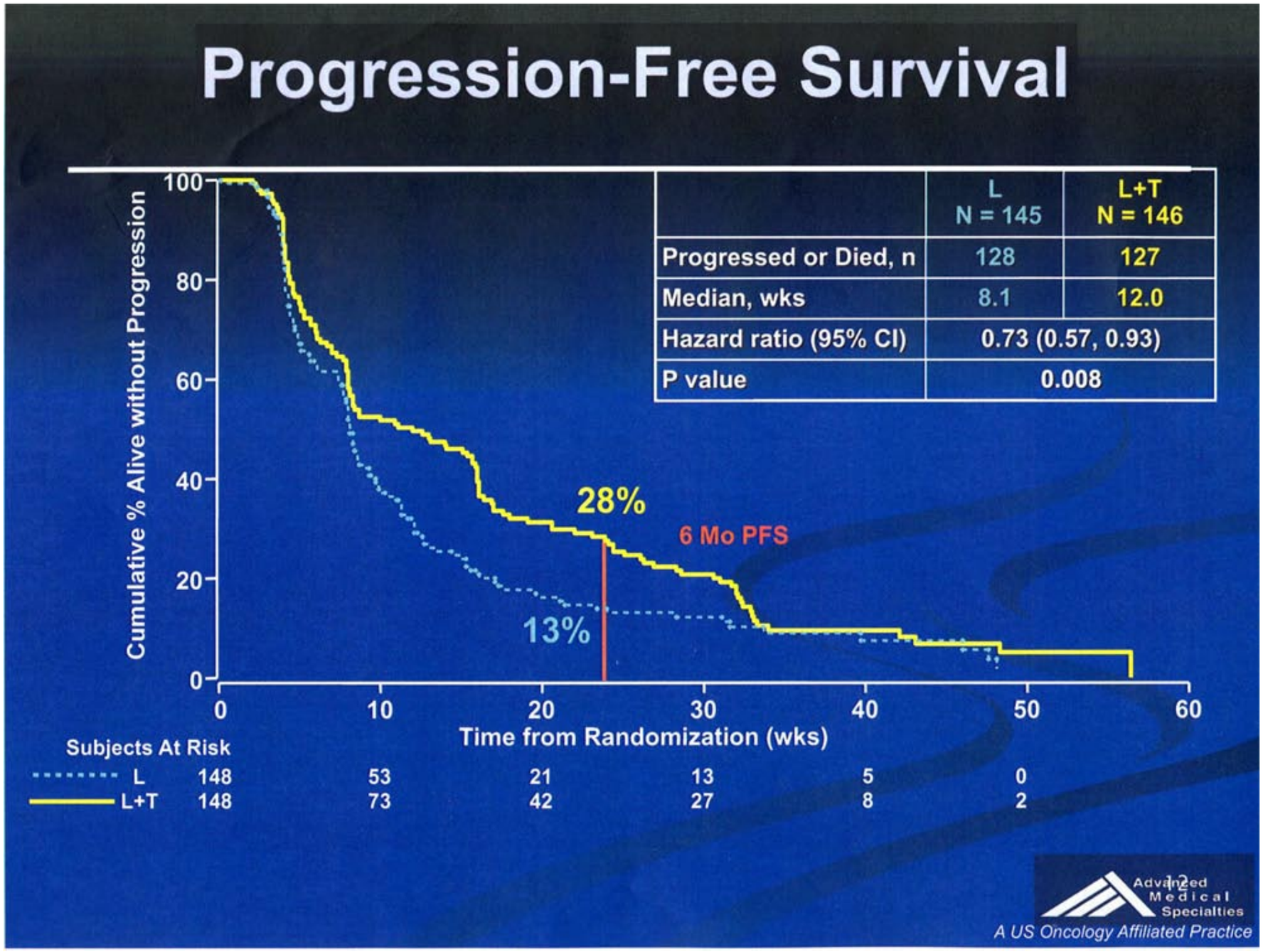






Ries LAG, Melbert D, Krapcho M, Mariotto A, Miller BA, Feuer EJ, Clegg L, Horner MJ, Howlader N, Eisner MP, Reichman M, Edwards BK (eds). *SEER Cancer Statistics Review, 1975–2004,* National Cancer Institute. Bethesda, MD, based on November 2006 SEER data submission, posted to the SEER Web site, 2007.

Here is the reason behind the flat mortality curves



This is a very depressing slide, and it is about something any oncologist deals with: RAPID EVOLUTION UNDER STRESS

The 800 lb. gorilla in this room is the rapid evolution of resistance to chemotherapy by cancer cells.

It occurs in some highly stressed microenvironment, cells evolve in response to stress imposed at some variable time scale from days to years, and come back resistant and deadly.

But is "natural selection", in simple the Neo-Darwinistic view, enough to explain the rapidity of this undoubted evolution?

I. WHY am I doing this:

B) Fundamental Questions in Evolution Dynamics (please don't hurt me)

My narrow view of neoclassical (Fisher) evolution modeling:

1) Successful mutations are random: $\Delta N = suN$

2) Mutation rates (u) are low: rate (u) of about 1/10⁹ mutations/basepair/generation.

$$u << 1$$

3) Most mutations are deleterious (reduce fitness). Selection coefficient very small:

$$s << 1$$

4) Evolution best studied in large numbers in big buckets, because of the low mutation rates and small selection coefficients. I think that is fundamentally wrong.

Regarding the mutation rate number u:

- I believe there are NO absolute numbers in biology, everything is context specific including mutation rates and where mutations occur.

- Evolution may be an emergent phenomena: it may be difficult to work strictly from a stochastic way UP to understand how complexity emerges.

- doing experiments under tightly controlled conditions, one of the powerhouse tools of the physicist, may entirely miss the point of emergent phenomena .

Here is an example:

E. coli has about 2.5×10^6 basepairs in its genome. "It has been sequenced and annotated": annotated means we know the genes.

Suppose we wanted to evolve in E. coli resistance an antibiotic which blocks a gene needed for replication.

Suppose a specific single-nucleotide polymorphism (SNP) (i.e., A to T) is sufficient to block the antibiotic from binding (extremely unlikely, in fact wrong).

If the normal error rate u is 10^{-9} bp generation, only 1 bacterium in 1000 has a mutation anywhere in its genome with each generation.

A single bacterium reproducing under high stress so that the population does not change will need 10^9 generations to escape. Hopeless.

Or, of course if you had 10^9 individuals in each generation, even without growth, one "Einstein" would have the magic mutation and take off in exponential growth. Or would s/he?

This "Einstein" has to compete with 1 billion morons for food and space.

1) The probability of fixation decreases with increasing population size N:

$$p_f \sim \frac{2s}{1 - \exp(-4Ns)}$$

2) The time to fix scales as 2N (big).

3) The time to lose scales as ln(N) (small).

So one has three basic problems:

1) The time to find mutation.

2) The low probability of fixing a mutation if there are many competitors.

3) The time to fix mutation if the number of competitors is large.

4) The time to lose a mutation is relatively small compared to the time to fix it as N increases.

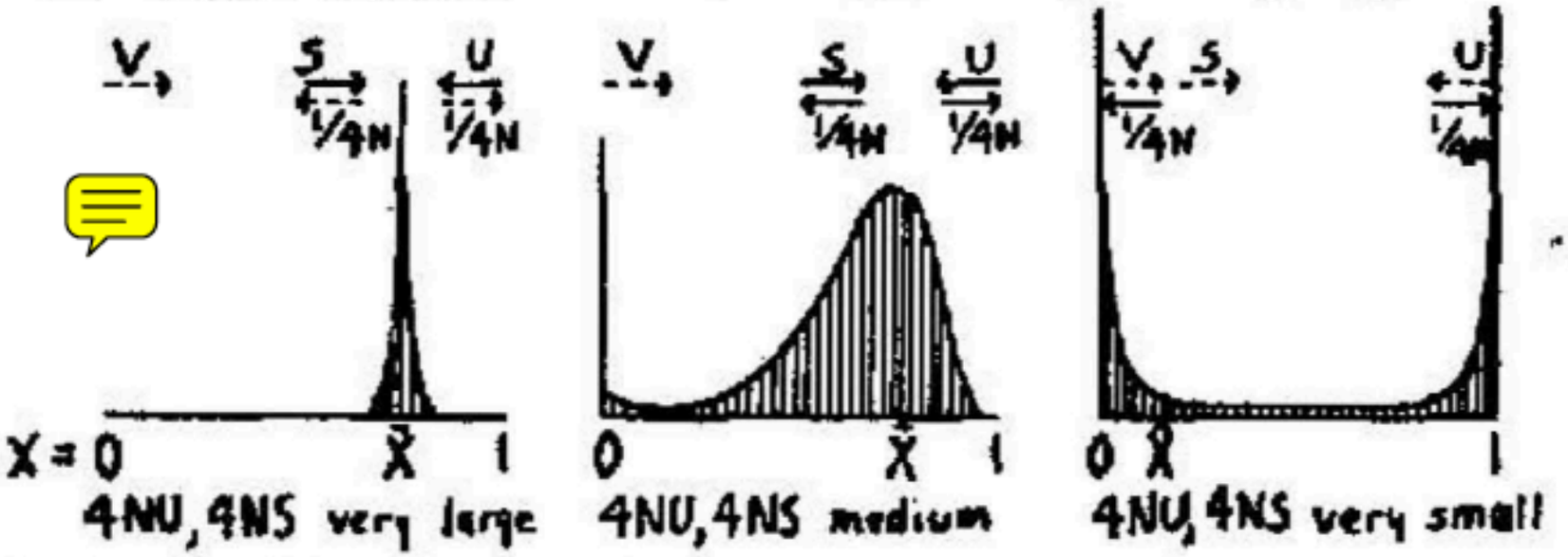
It is legitimate to ask: Does the random model of neo-Darwinism really explain the speed of evolution in the real (that is, highly heterogenous) world outside the ivied towers of academe or the rarified world of theory??

In the 30's, theoretical evolutionary biologists, in particular Sewall Wright struggled with how to put a mathematical framework on Darwin's vague and hand-waving "survival of the fittest" explanation for the increasing complexity of life.

Distribution of Gene Frequencies

A. Whole Species

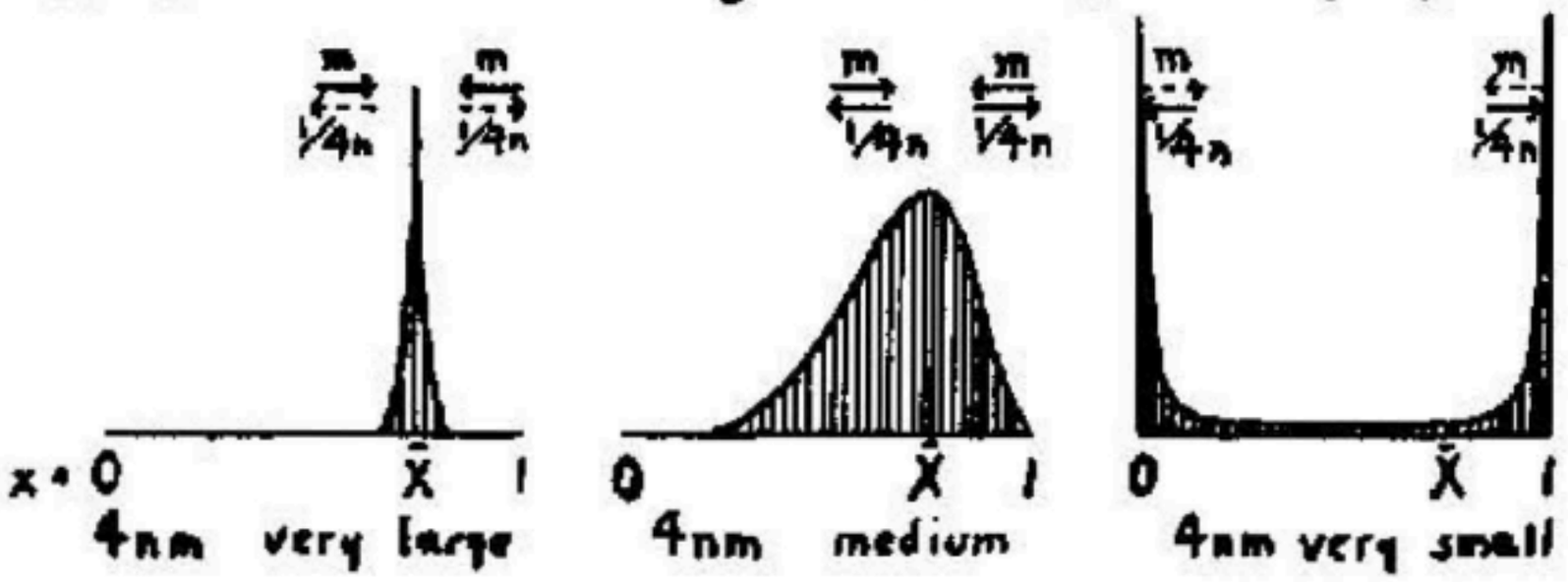
$$Y = Ce^{4NSX} X^{4NV-1} (1-X)^{4NU-1}$$



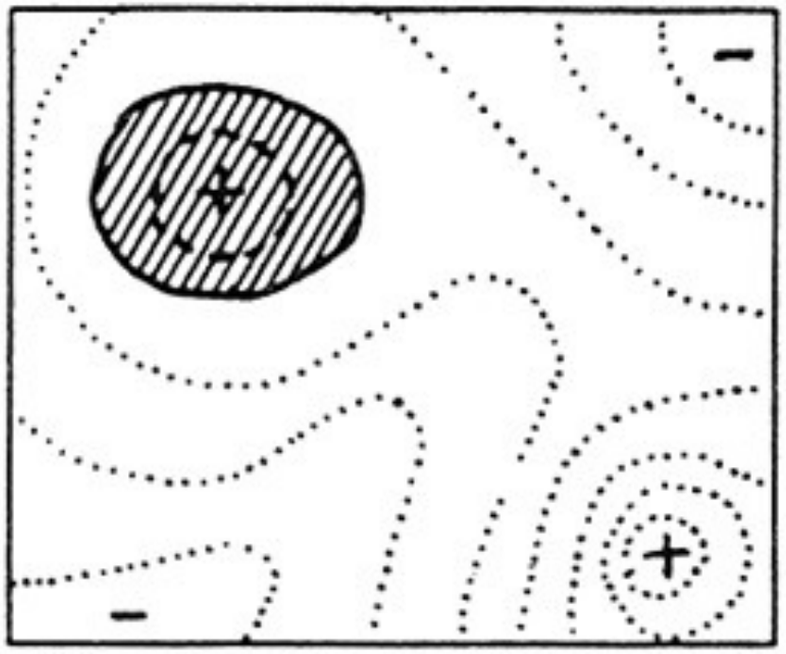
X	gene frequency
Y	probability
C	coefficient
N	population number
S	selection coefficient
V, U	mutation rates to and from gene, respectively, per generation

B. Local Race

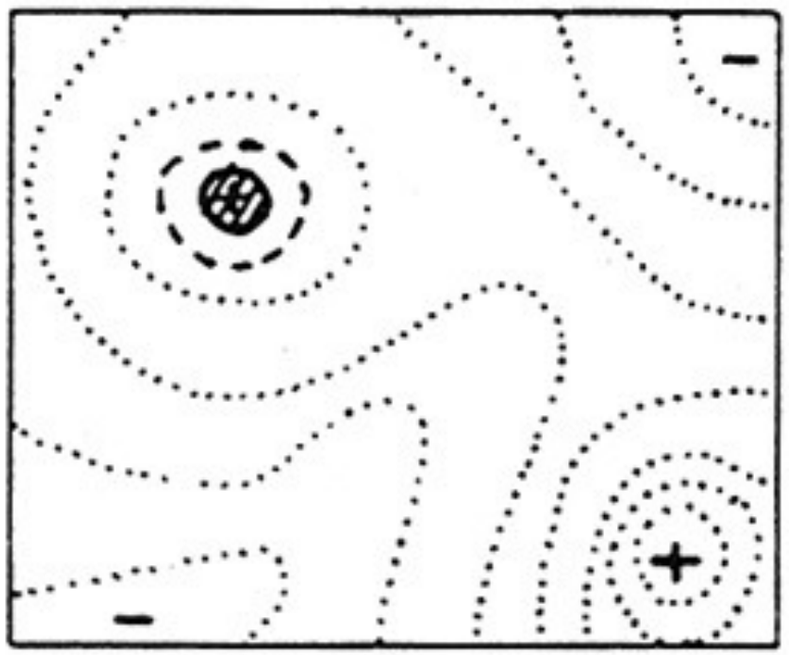
$$y = Ce^{4nsx} x^{4nm\bar{X}-1} (1-x)^{4nm(1-\bar{X})-1}$$



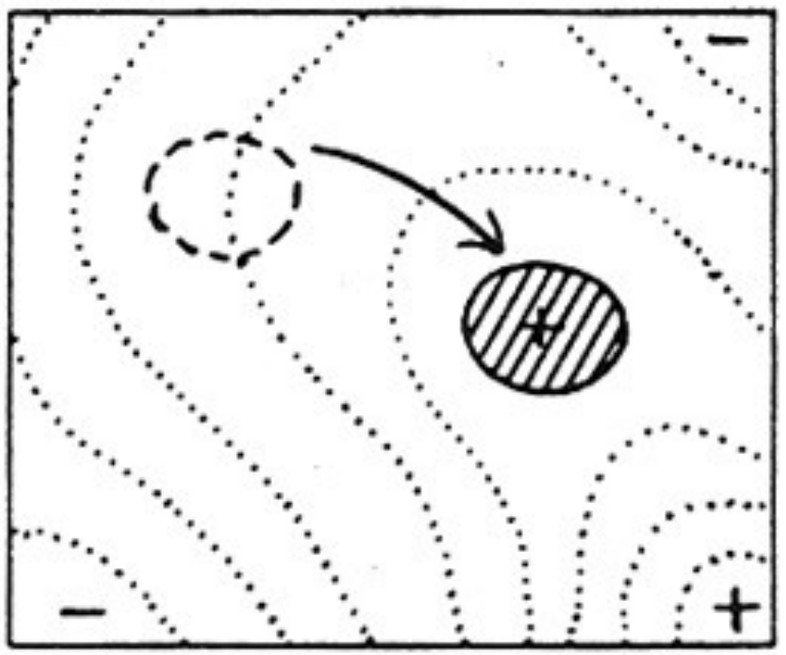
x	gene frequency
y	probability
C	coefficient
n	population number
s	selection coefficient
m	population exchange with rest of species



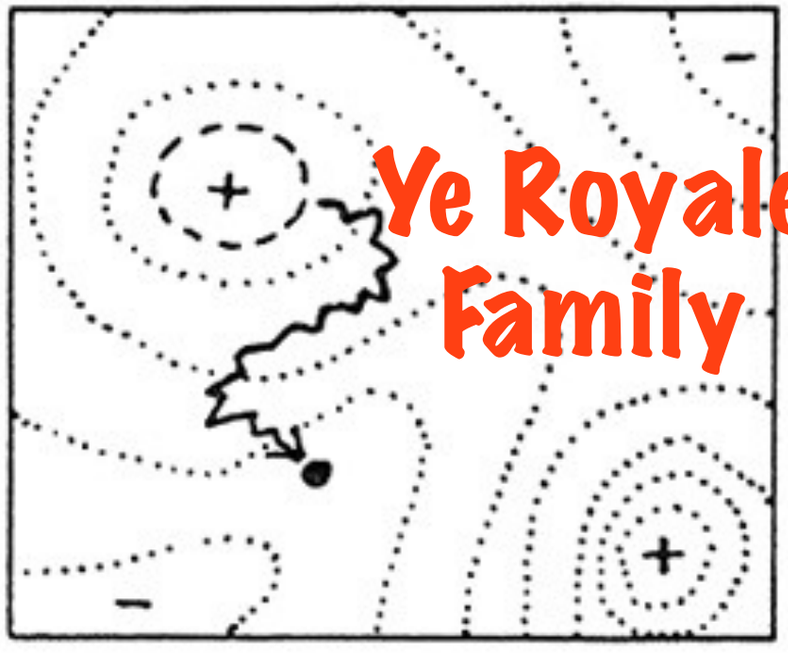
Increased Mutation
or reduced Selection
4NU, 4NS very large



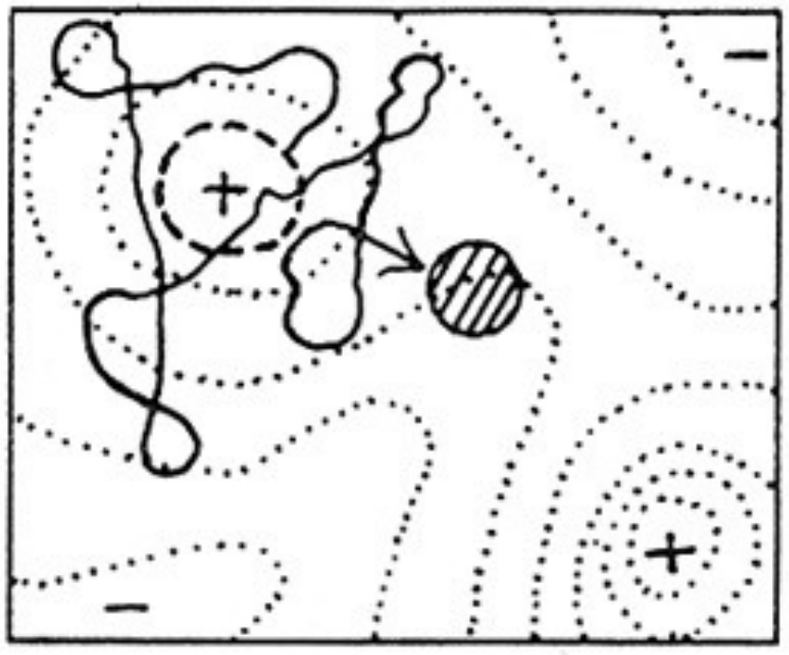
Increased Selection
or reduced Mutation
4NU, 4NS very large



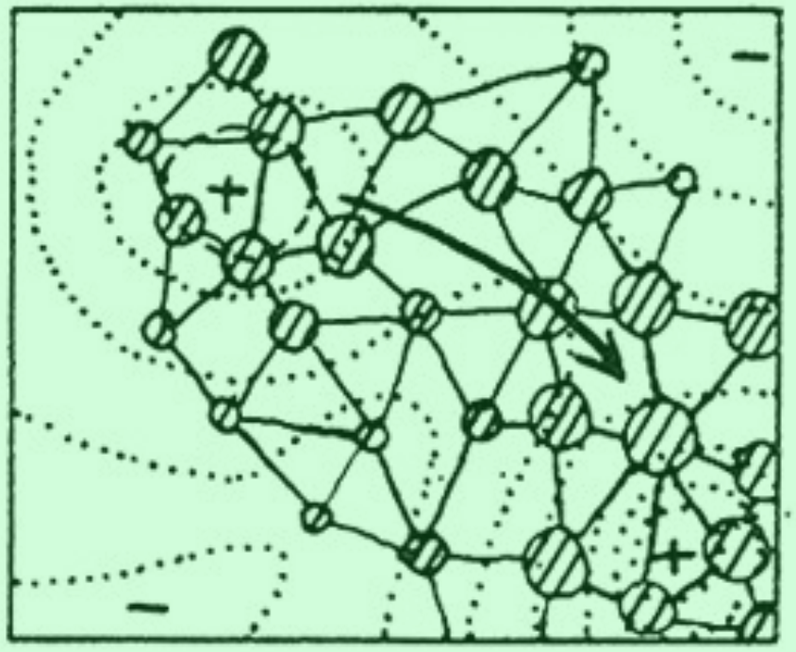
Qualitative Change
of Environment
4NU, 4NS very large



Close Inbreeding
4NU, 4NS very small



Slight Inbreeding
4NU, 4NS medium



Division into local Races
4nm medium

Ye Royale Family

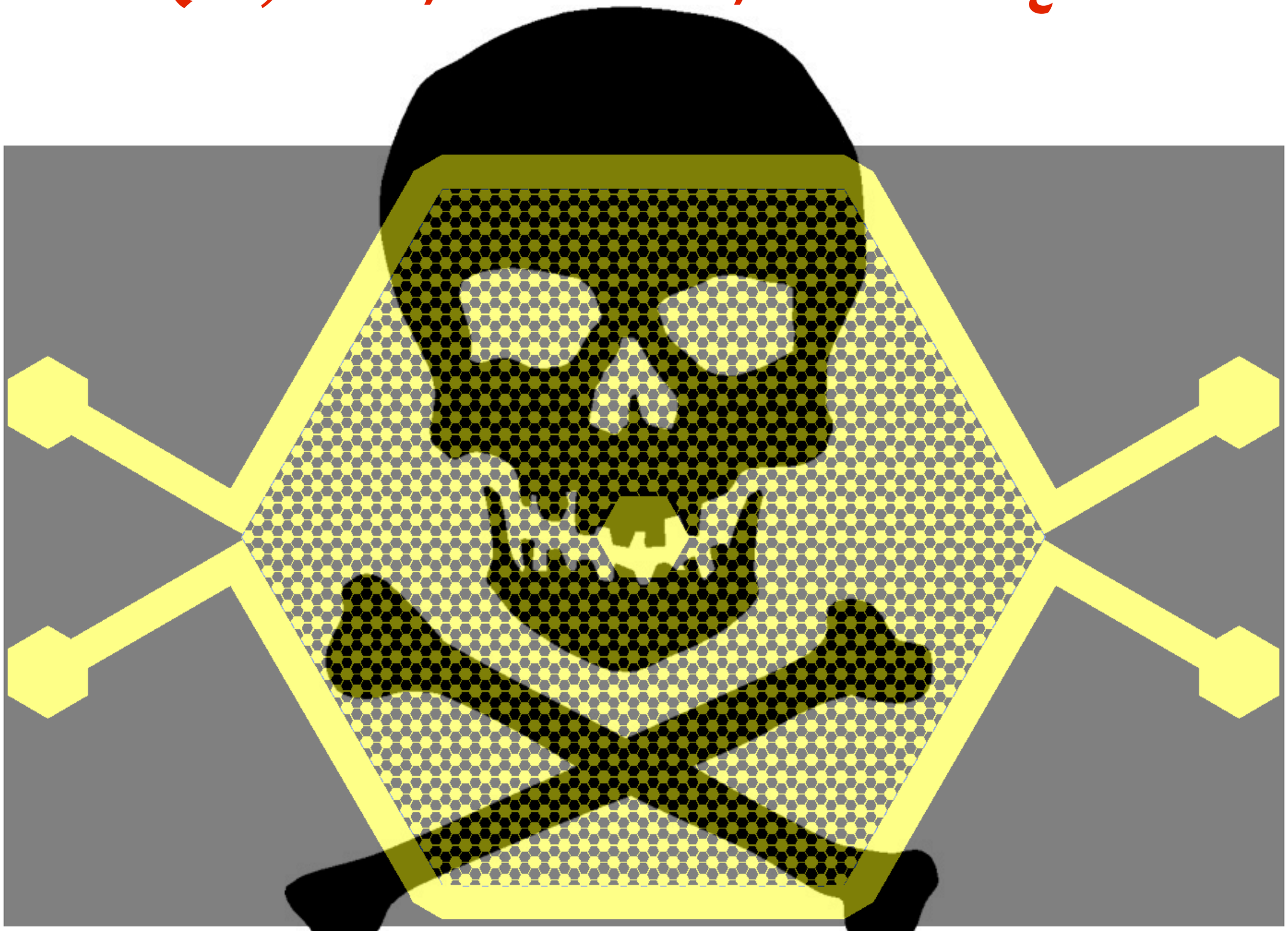
Sewall Wright, 1932

III. The basic experiment to accelerate evolution without large flasks and 20 Years of time using physics and microfabrication.

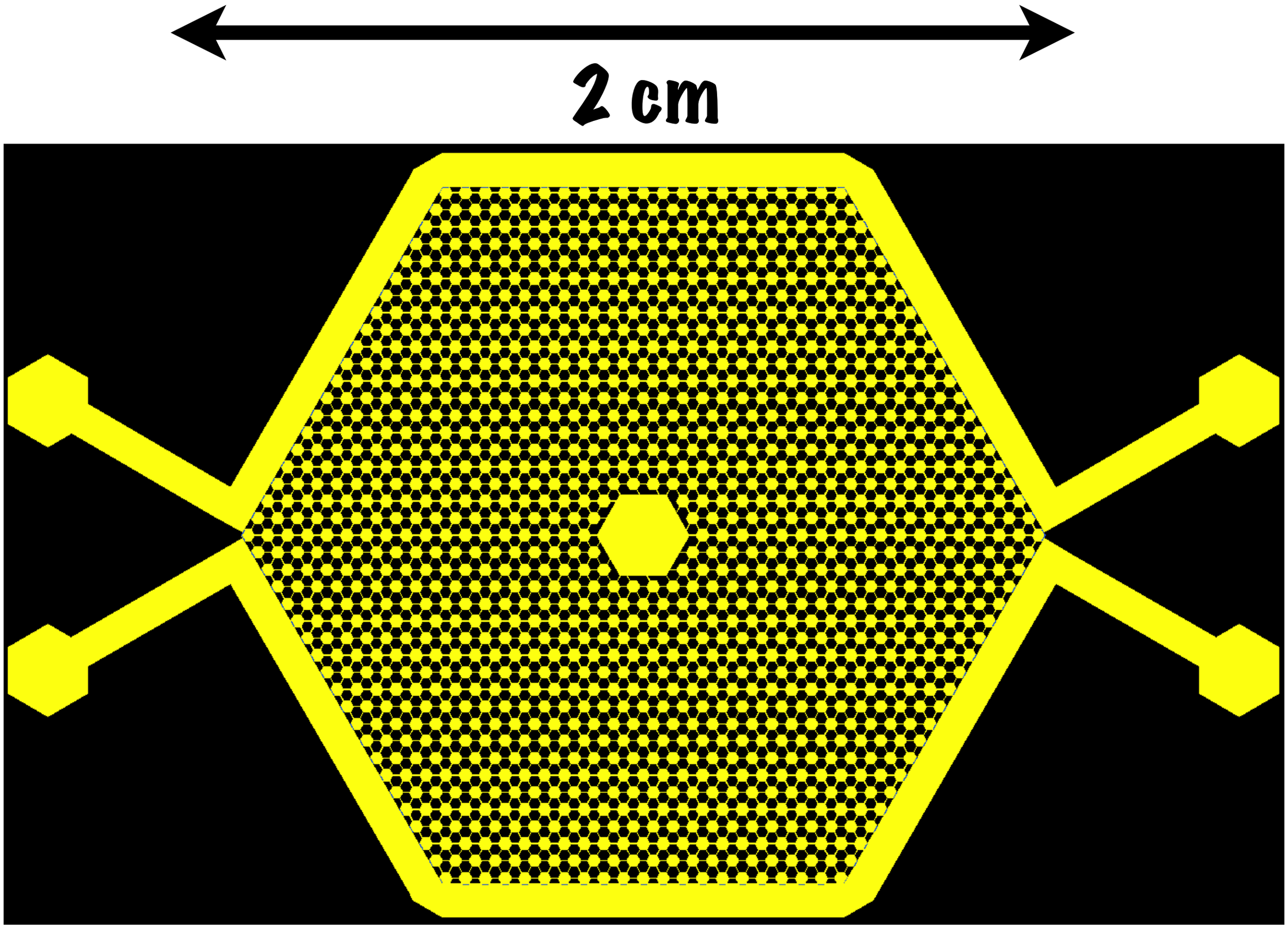
“So, what I will do is present this in reverse order, because the technology should become an important tool to microbiologists, but Austin has poisoned the well so thoroughly that no one will touch it until it is invented independently of him. “

Some strange sort of koshering process by a rabbi-biologist?

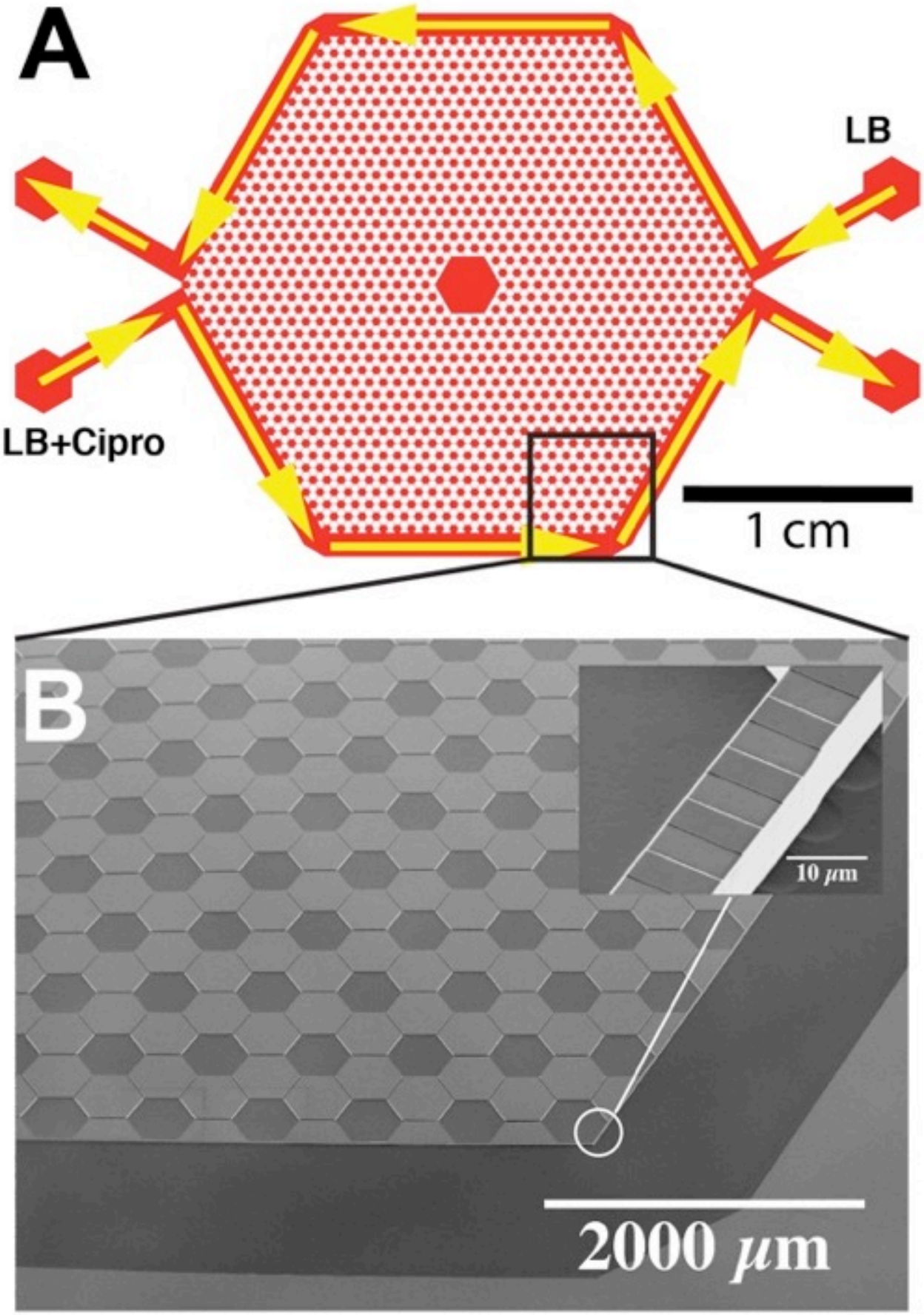
(A) The Death Galaxy

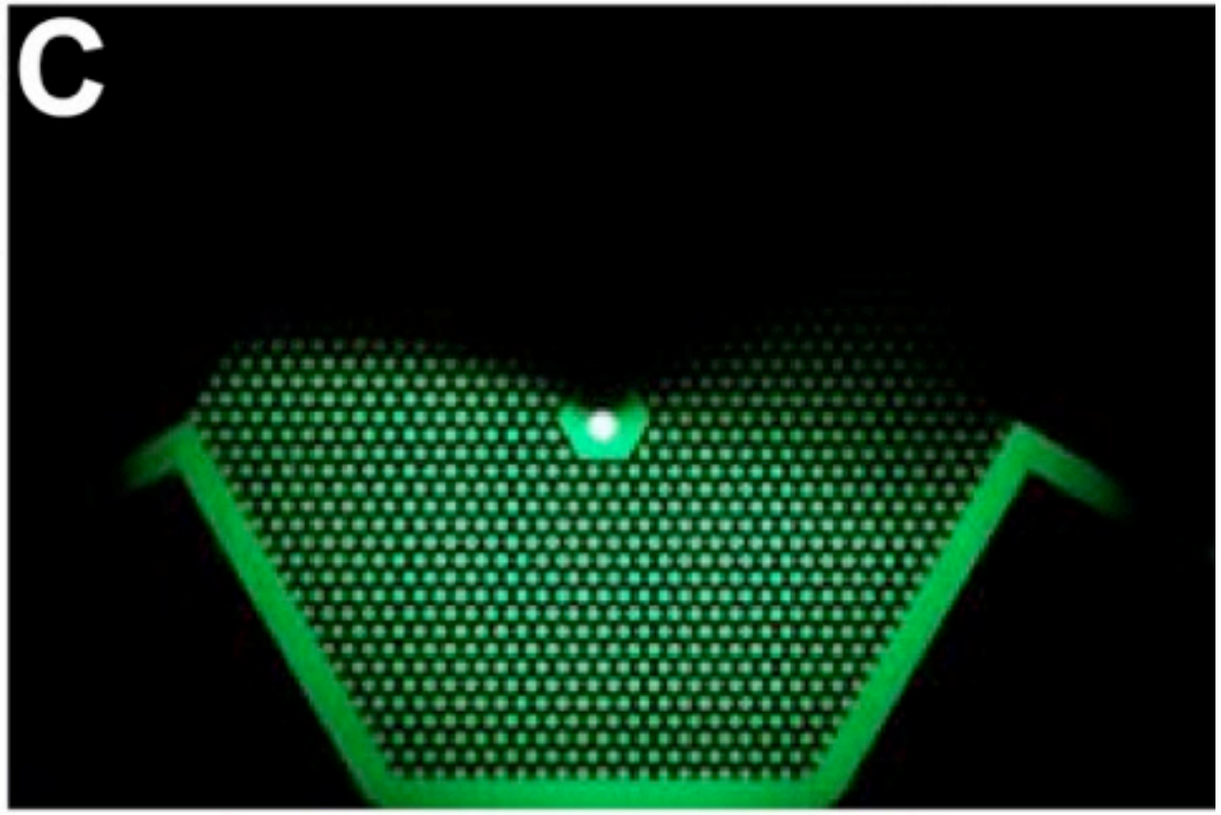


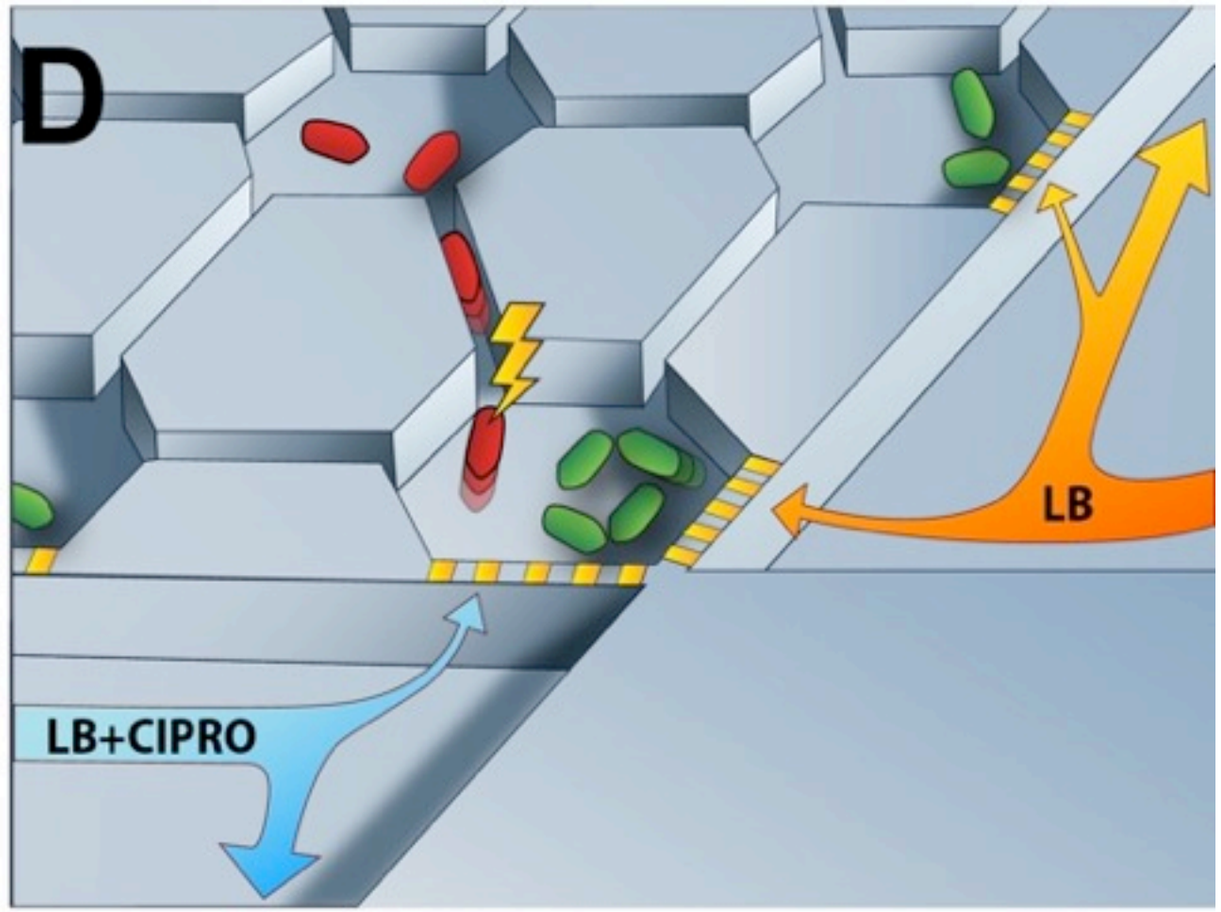
(designed in Hong Kong)



My attempt to realize Wright's Fitness Landscape


A
LB
LB+Cipro
1 cm
B
10 μm
2000 μm


C

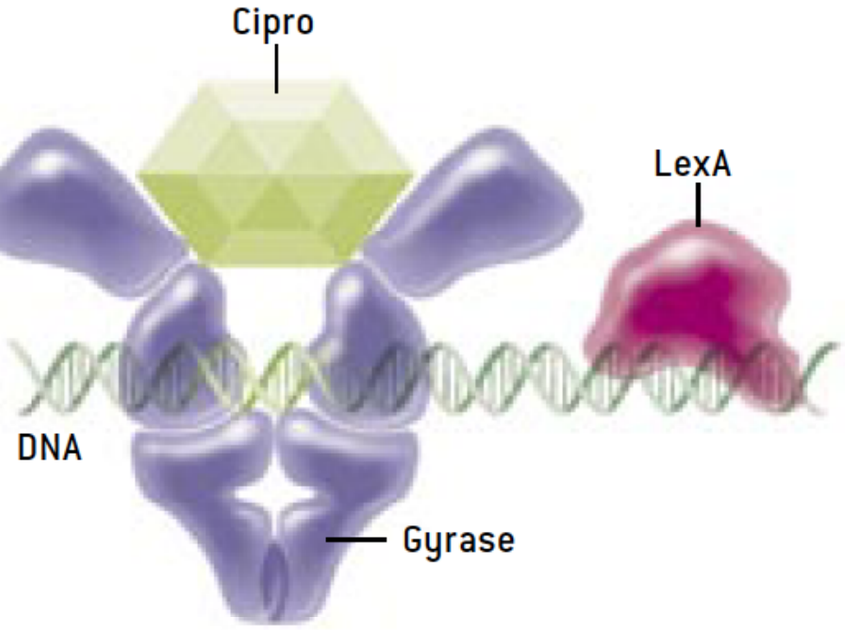

D
LB
LB+CIPRO

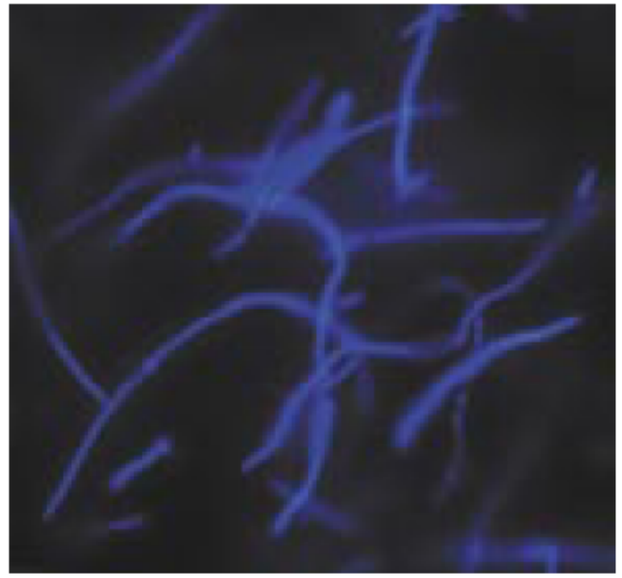
Cipro: a genotoxic antibiotic

Rapid-fire mutations in *Escherichia coli* bacteria can undermine the effectiveness of ciprofloxacin (cipro), an antibiotic that is increasingly being prescribed by physicians.

Cipro's Action

Cipro usually harms bacteria by binding to an enzyme called gyrase and preventing it from functioning properly

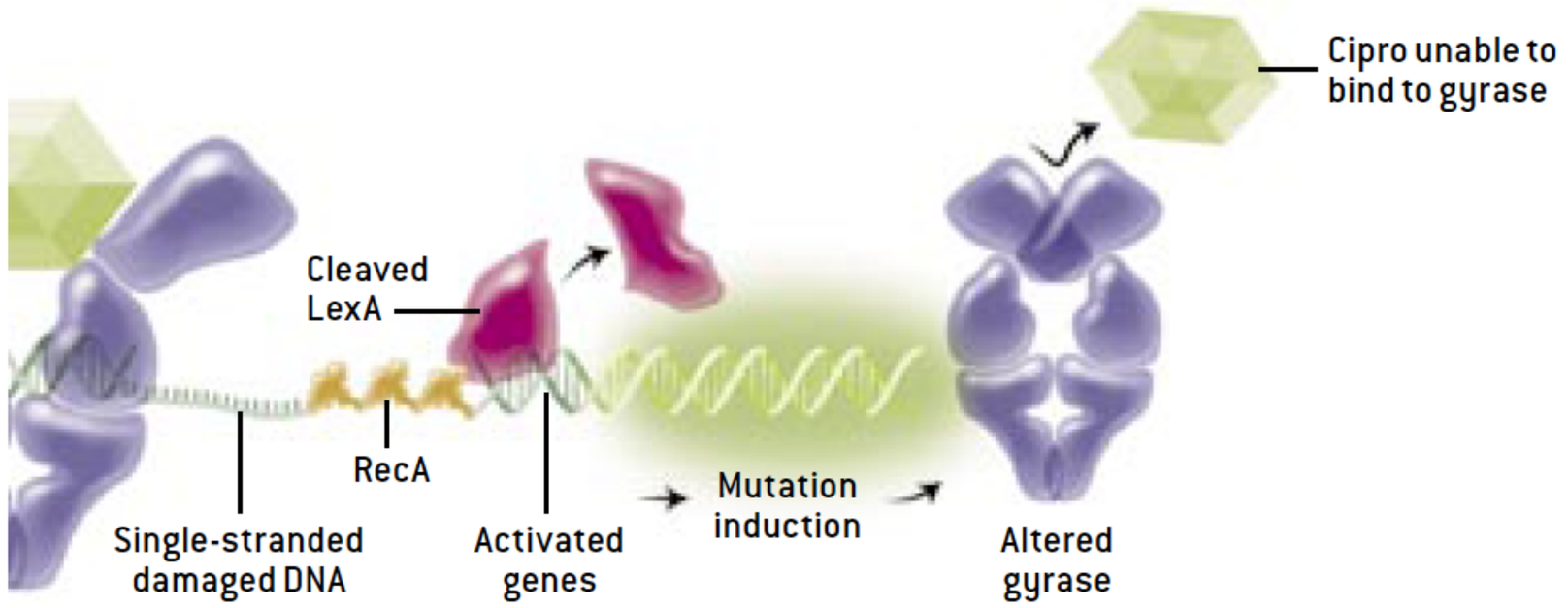




DNA of *E. coli* (*shown above*) cannot replicate when it is exposed to cipro.

How Resistance Arises

Resistance is initiated when *E. coli* responds by generating single-stranded DNA. Individual molecules of another protein, RecA, then line up in a chain and attach to the single-stranded DNA. RecA facilitates cleavage of a regulatory protein, LexA. This change frees a set of formerly repressed genes to induce mutations elsewhere. The mutations end up blocking cipro's binding to gyrase, thereby preventing the drug from working



Note: in spite of the attacks on me (physicist on physicist crime!) the initiation of high mutation rates by an error-prone polymerase as a result of antibiotic blocking of a gyrase is not something I made up, it is well known in the literature (if you read the literature).

The estimate is that the error rate u* increases by up to 4 orders of magnitude, to 10^{-5} from 10^{-9} per bp per generation.

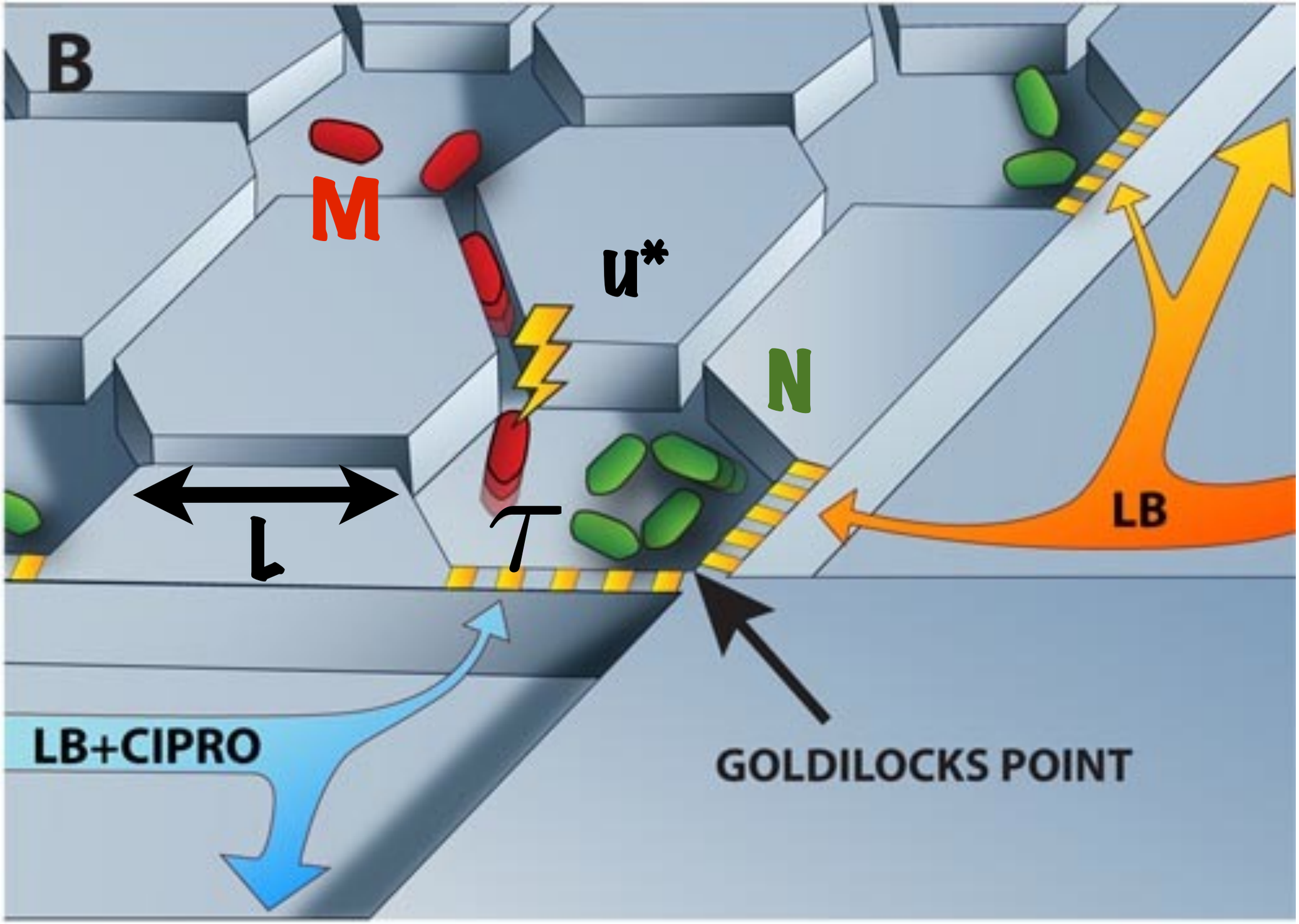
You CAN argue about where the mutations occur (stochastic or not), and there are certainly "hot spots" for mutations observed.

Spatial heterogeneity coupled with motility coupled with stress can accelerate evolution.

Terry Hwa (UCSD Physics) and Rutger Hermsen (UCSD Physics, now Delft Kavli Institute Nanotechnology)

Sources and sinks: a stochastic model of evolution in heterogeneous environments, Hermsen, R., Hwa, T., Phys. Rev. Lett., 105: 248104 (2011)

Goldilocks Points: Being at the Right Time at the Right Place



Evolution consists of 3 steps:

1) The mergence of a mutant (M) in the presence of stress on wild-type N, stress accelerates the mutational rate (u*).

2) The movement of the emergent mutant M to a region of HIGHER stress where the selective advantage is enhanced ($\nabla N_e \cdot \vec{v}$)

3) Successful competition in a microhabitat against small number of competitors.

I also think these are the 3 steps to cancer.

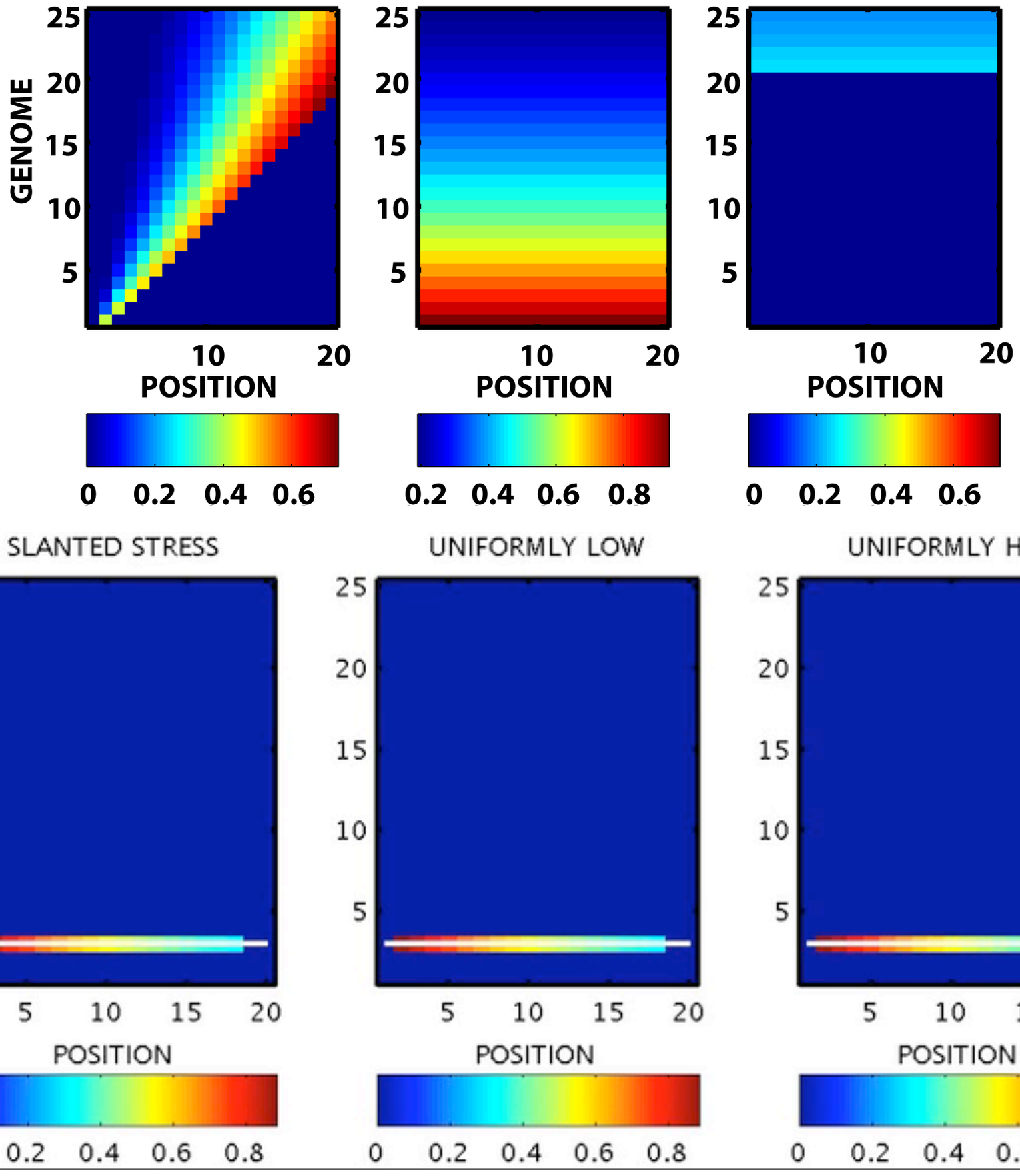
Modified form of Fisher-Kolmogorov Eq.:

$$\frac{\partial m}{\partial t} = \mu^*(x,y)(n + \nabla n \cdot \vec{L}) + r_m(x,y)m(1 - \frac{m+n}{K_h}) + \frac{L^2}{2\tau}\nabla^2 m$$

$$\frac{\partial n}{\partial t} = r_n(x,y)n(1 - \frac{m+n}{K_h}) + \frac{L^2}{2\tau}\nabla^2 n$$

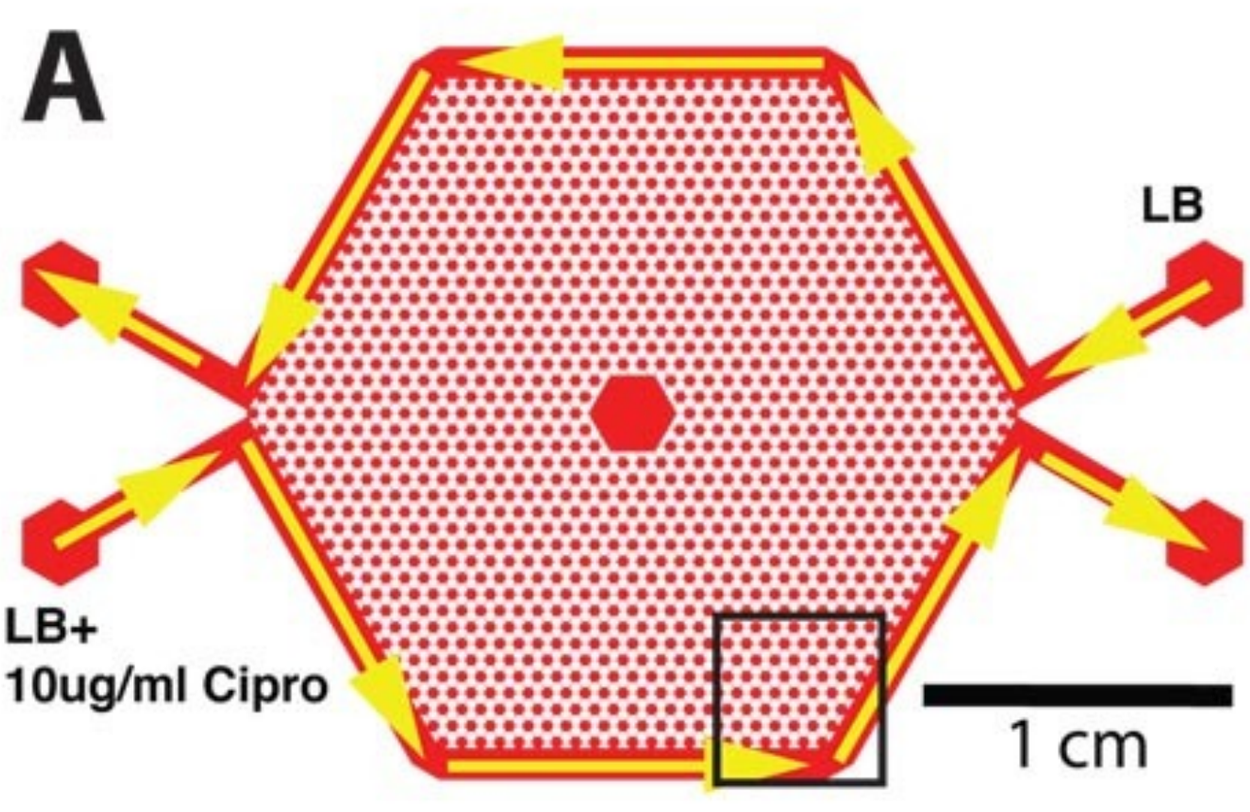
The prediction is that the emergence time will scale inversely with the initial doping number, in a nonlinear way. A complex equation.

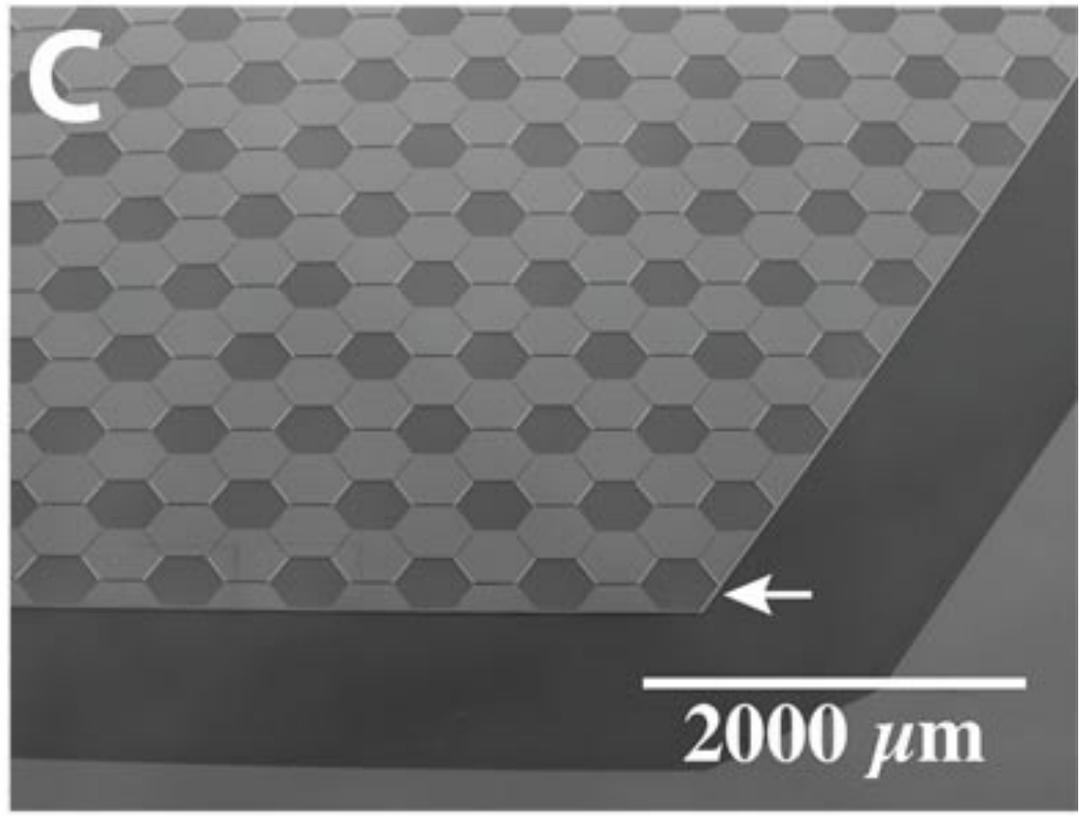
In collaboration with Terry Hwa

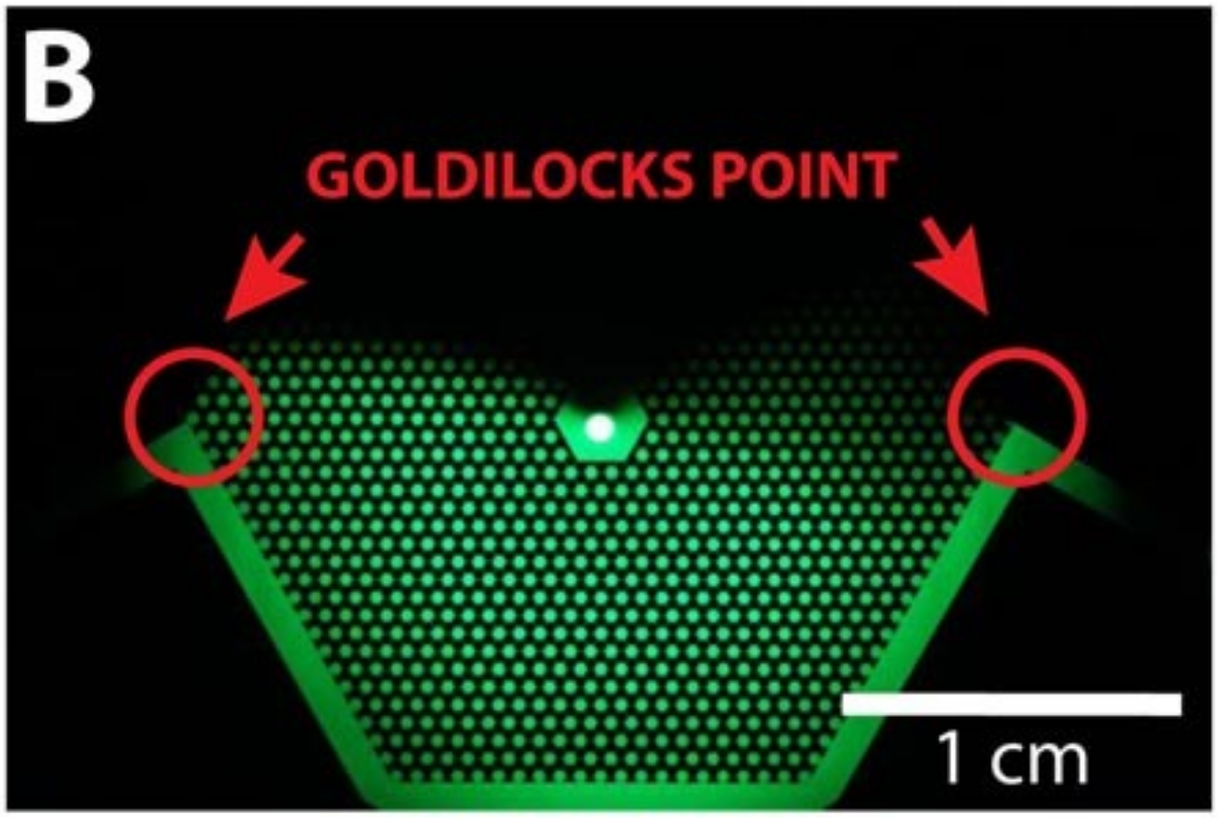


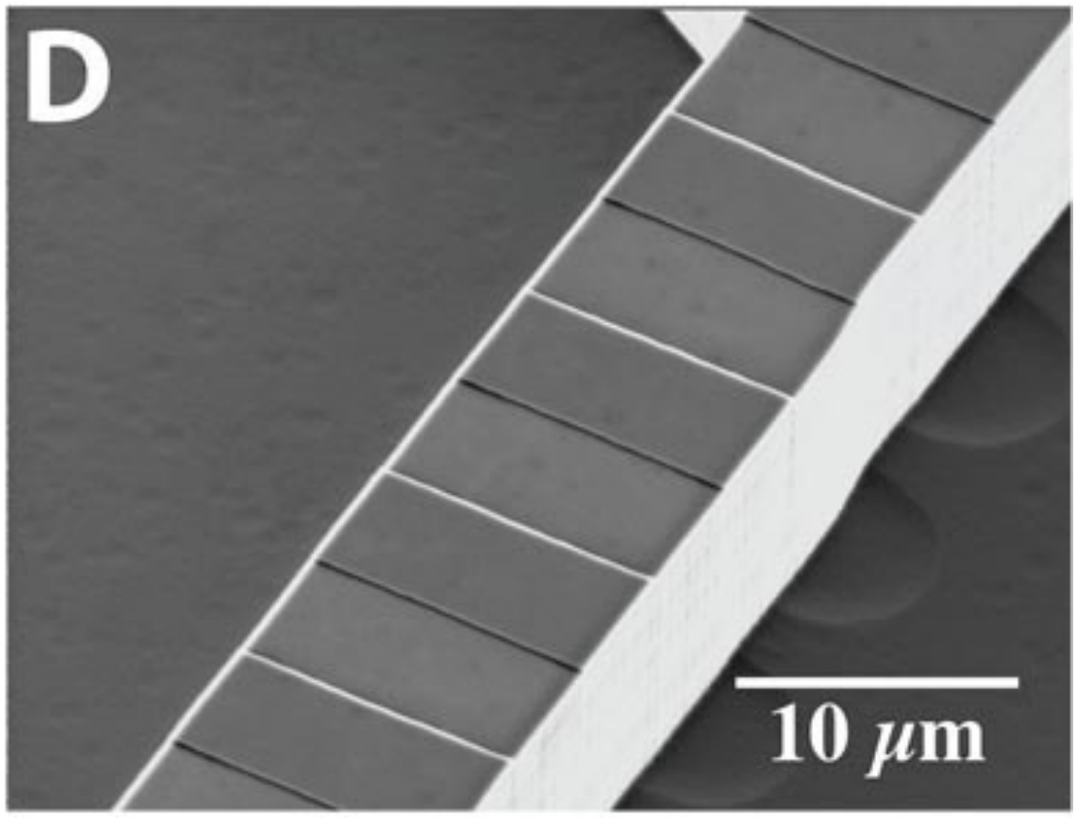
(B) First experiments

“Way to avoid using the word "crackpot".”


A
LB
LB+
10ug/ml Cipro
1 cm


C
2000 μm


B
GOLDILOCKS POINT
1 cm


D
10 μm

NOTE! I can create HUGE stress gradients using micro-nanofabrication technologies here, gradients that do NOT exist within the sterile plastic tube confines of your typical microbiology laboratory.

The stress gradients are due not only to the antibiotic gradient, but also the metabolic gradient, for not much food enters into this device.

It resembles a tumor architecture, highly toxic in the core.

Now, inoculate bacteria into the antibiotic Cipro gradient!

Can our bacteria evolve resistance rapidly? Or are there persisters already resistant to Cipro?

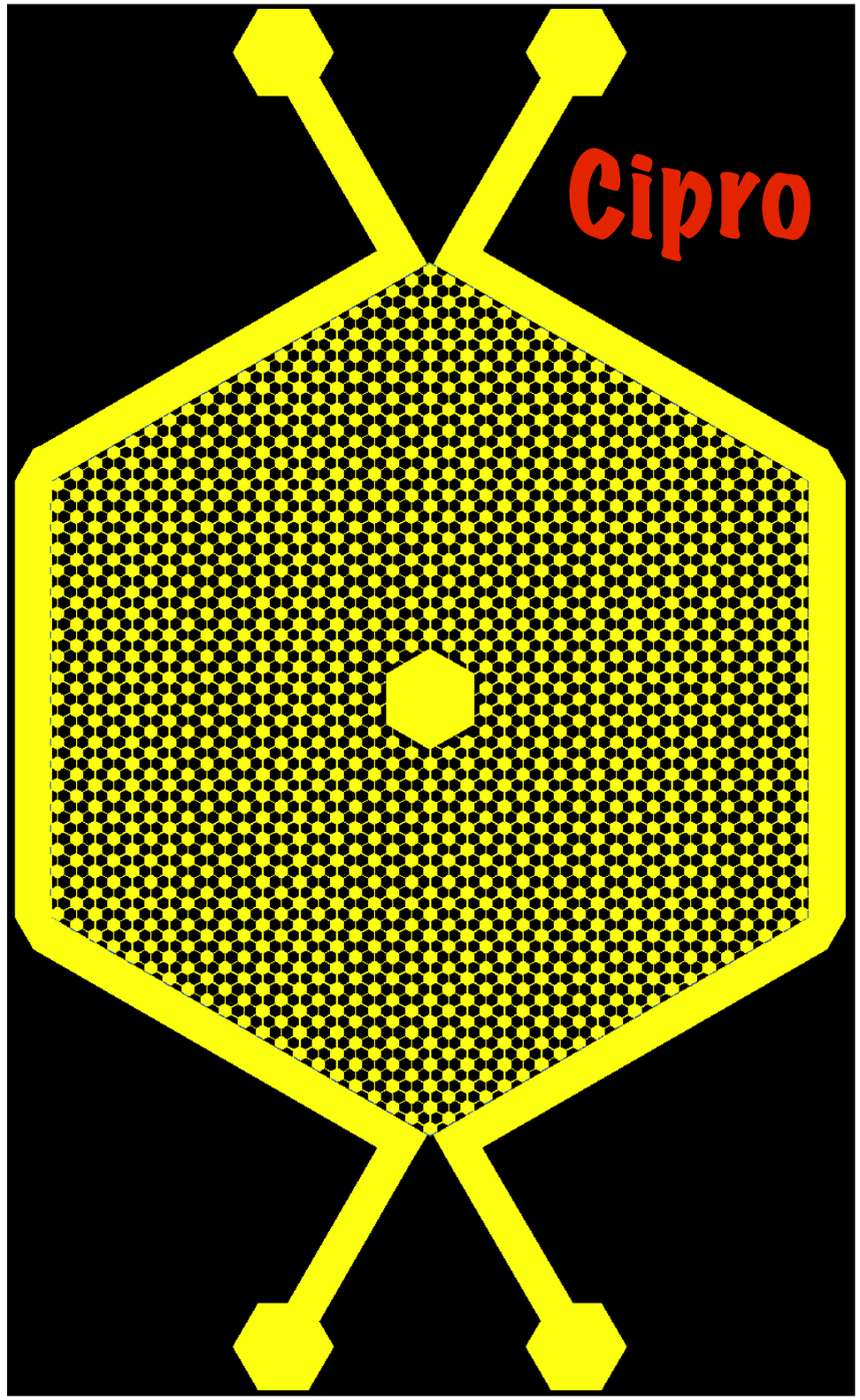
If the gradients are too weak, will evolution to resistance occur?

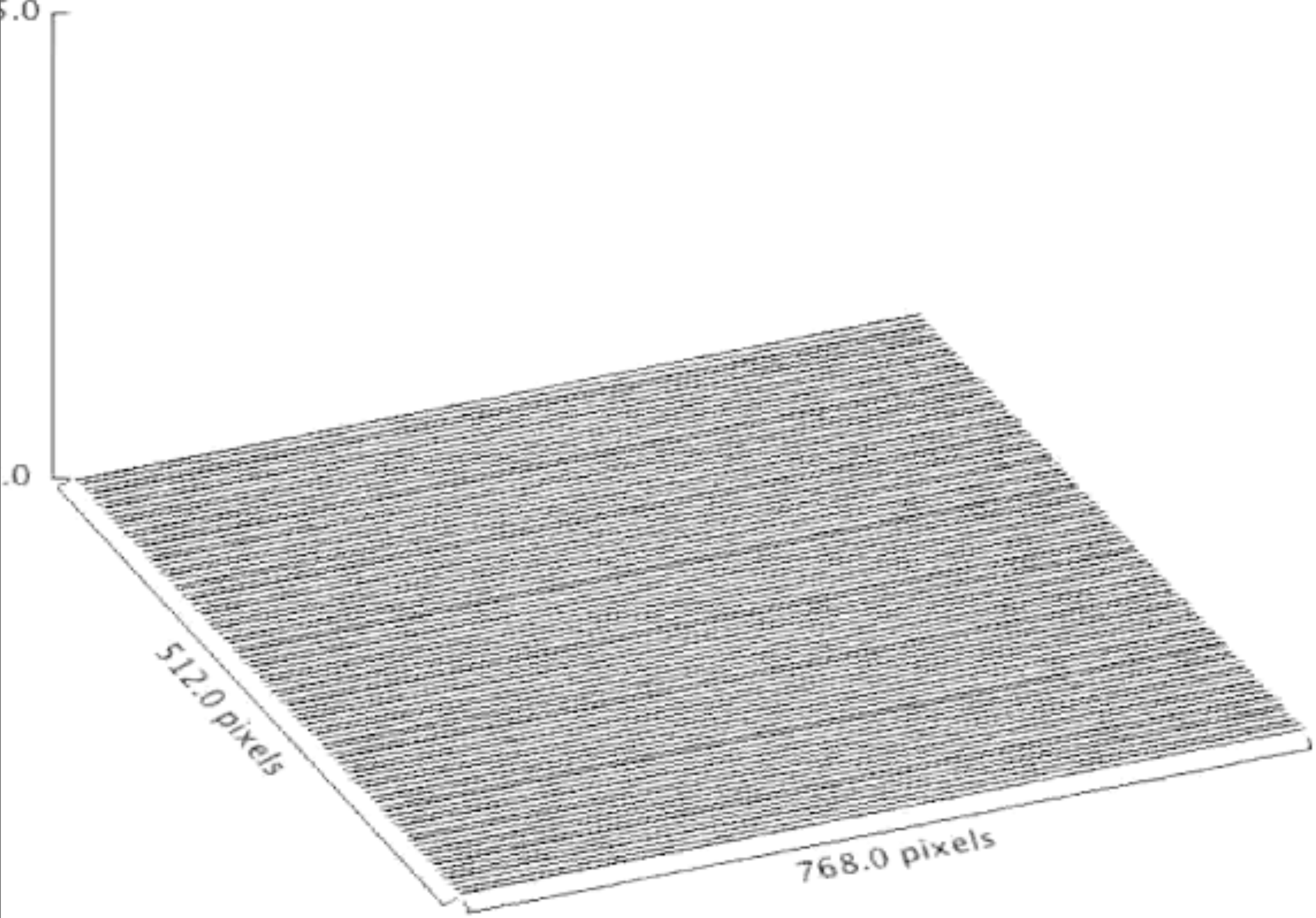
If evolution occurs, how quickly does it occur?

Will the same thing happen if you simply run the bacteria in separate wells without motility?

Cipro

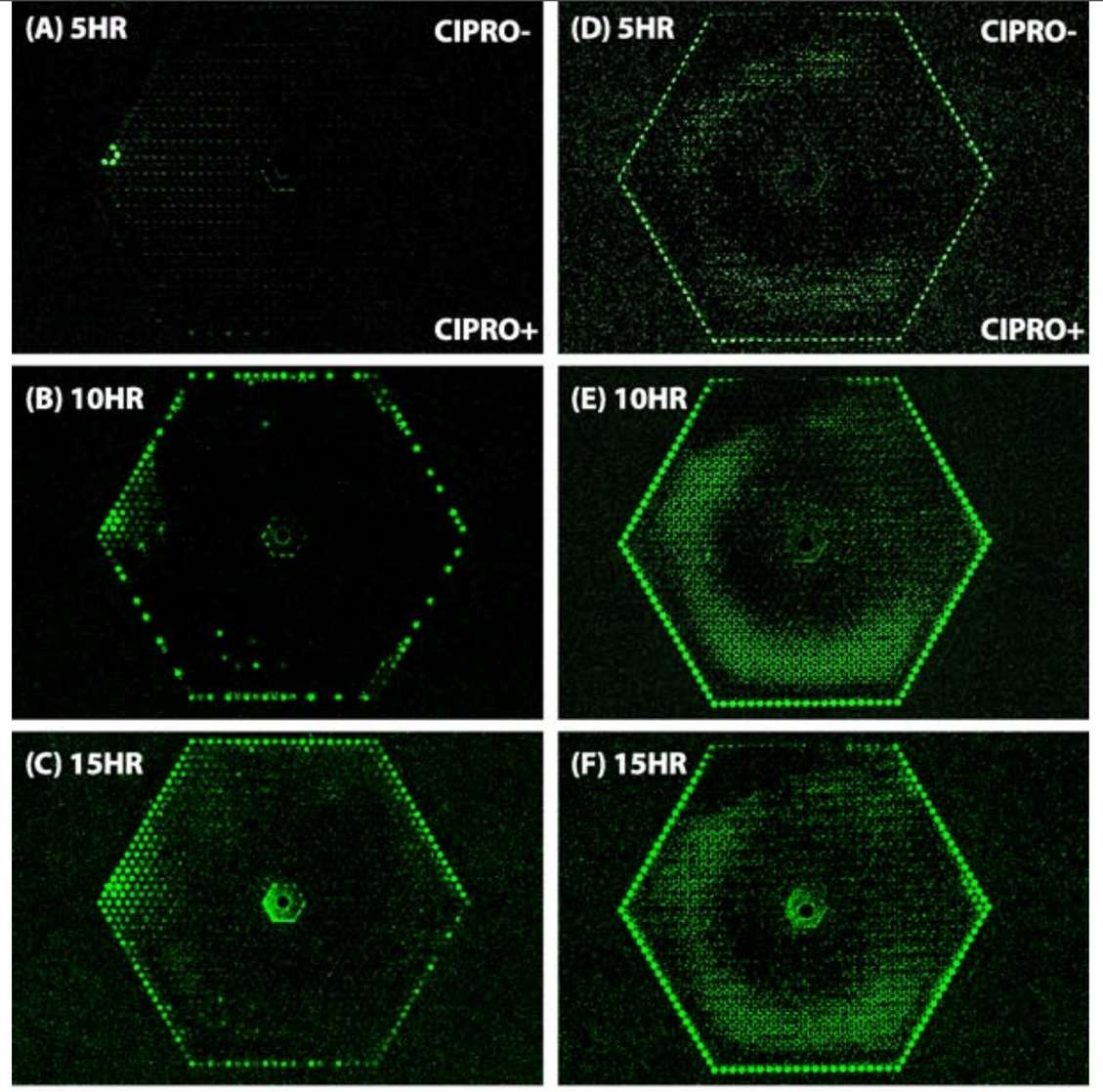
10^6 initial bacteria



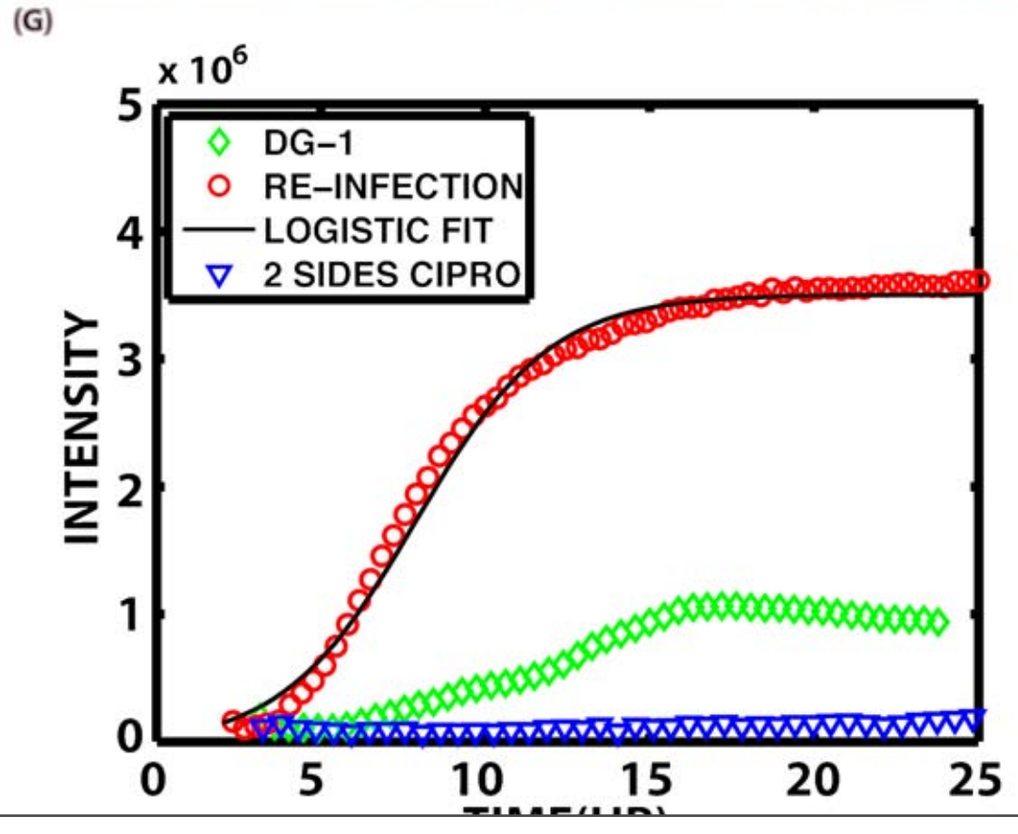


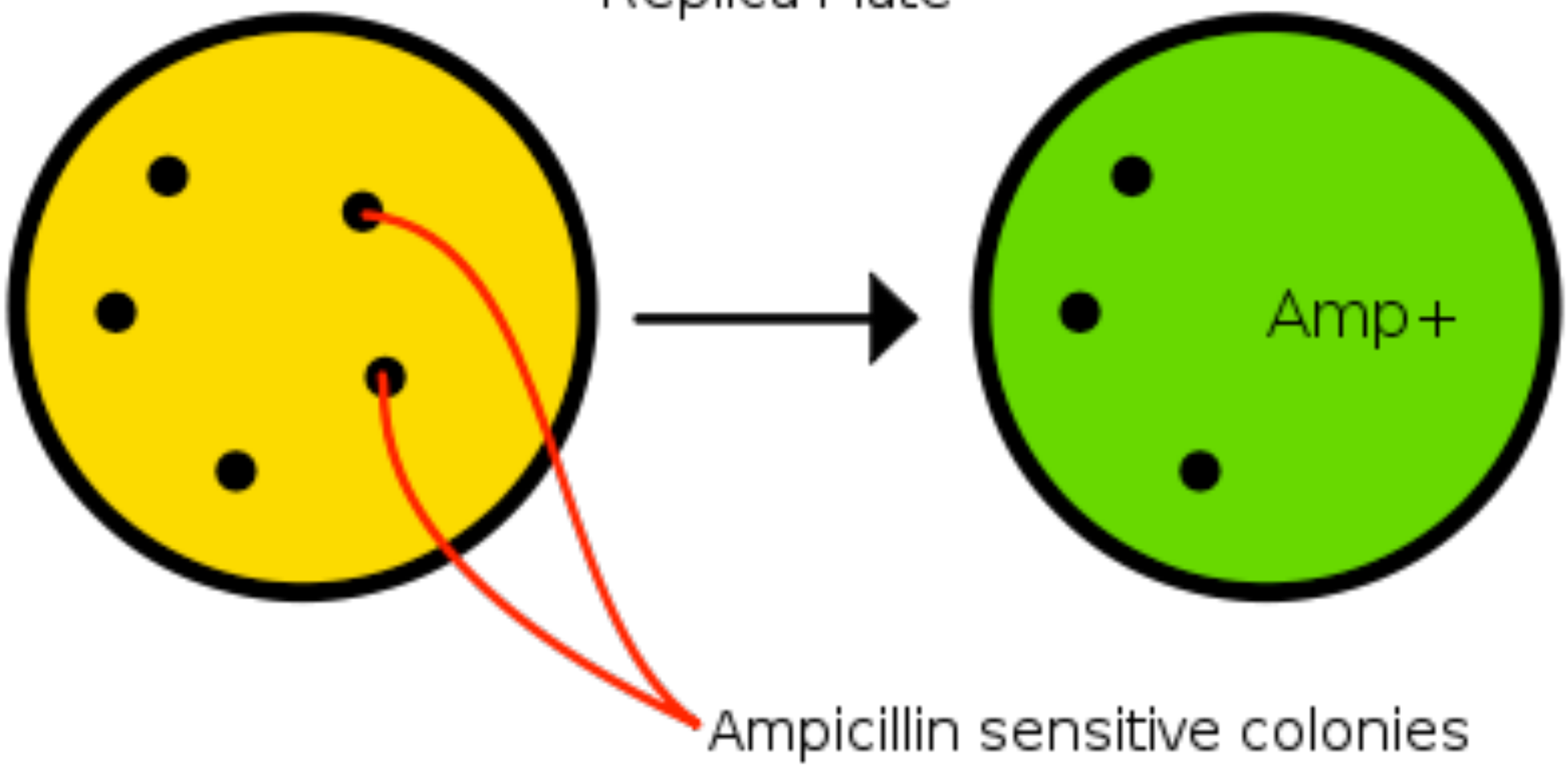
Friday,25 March, 11

What happens if you reinoculate the mutant bacteria into a DG? Is the phenotype now different?



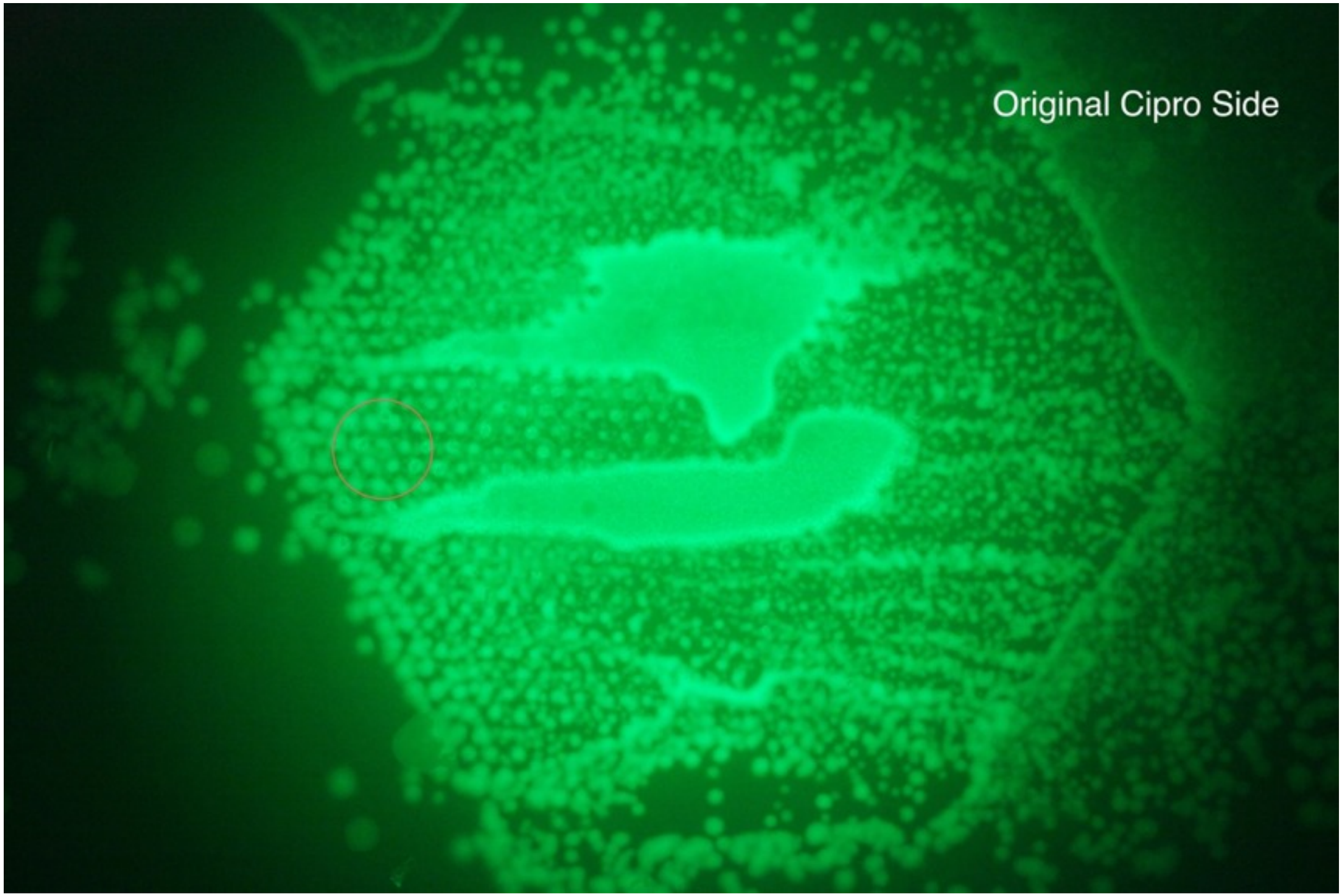
What happens if you remove the gradient?





Do we see Cipro resistance when we replica plate our Death Galaxy onto a +Cipro plate?

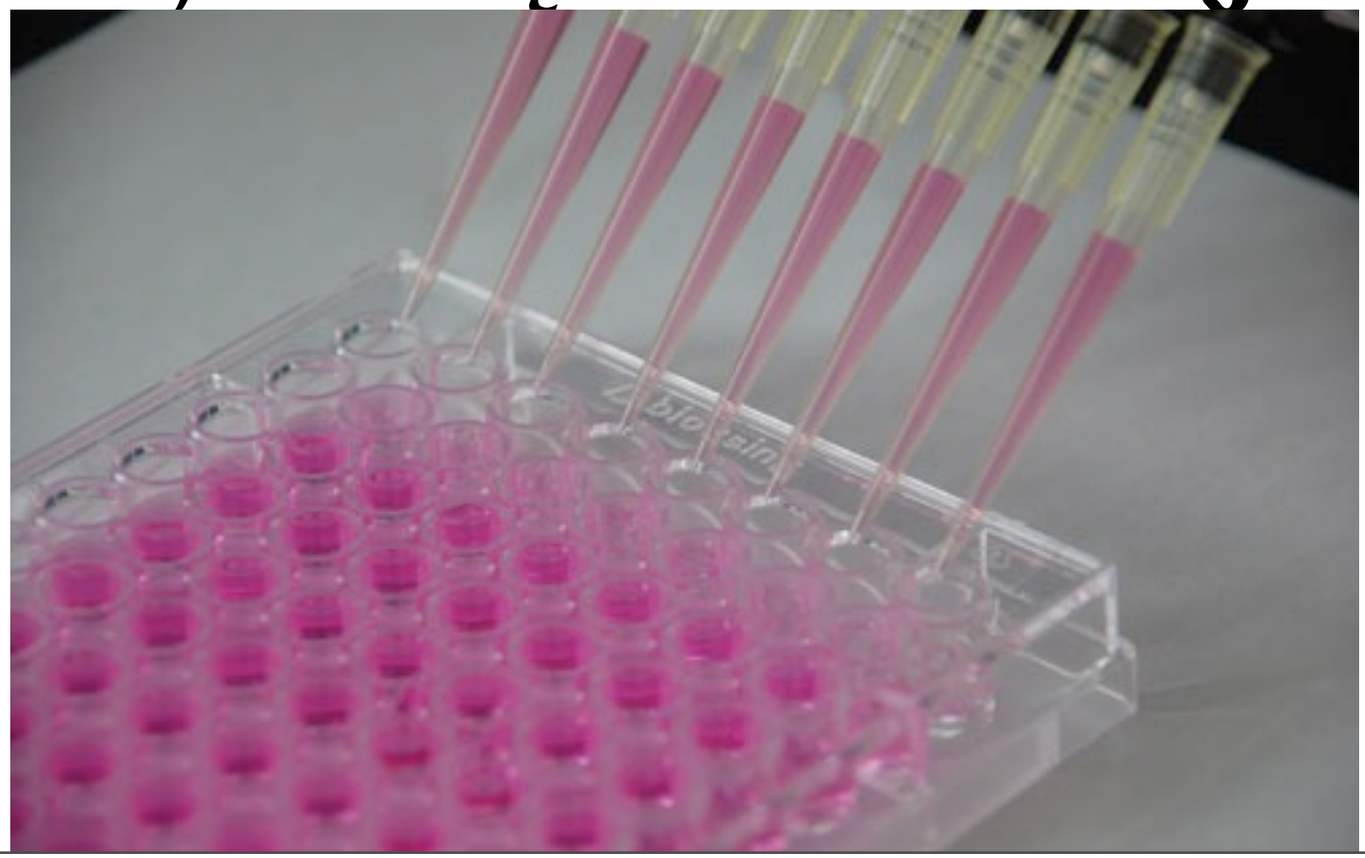
Yes!



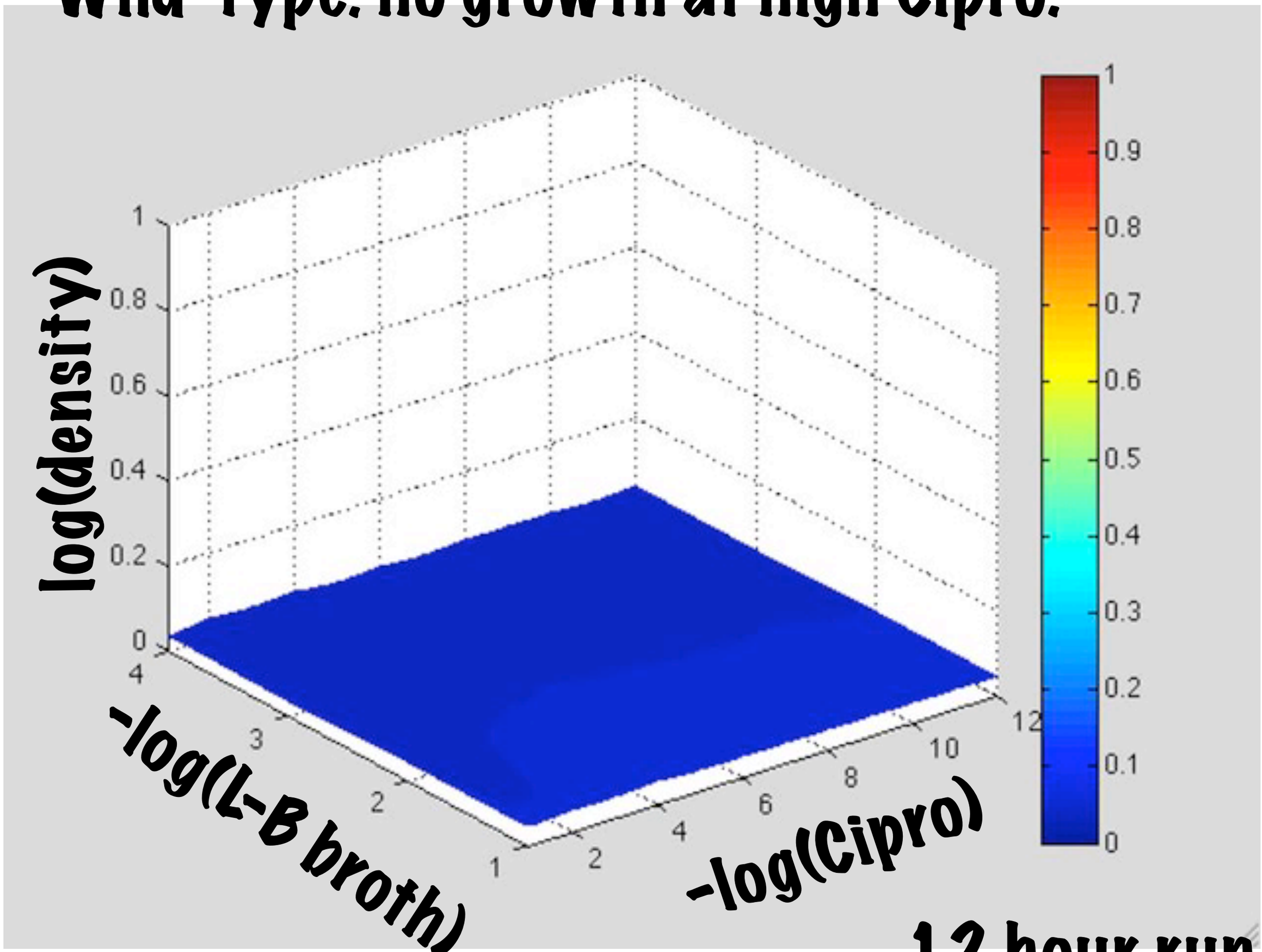
10 ug/ml + Cipro agar plate

4th challenge: If you get rid of the gradients but apply the same stress conditions in test tubes, does evolution stop (or slow down greatly?)

Suppose I use a 96 well plate and put bacteria in each one of those little wells with a matrix of food and antibiotic, no spatial gradients. It's like the Death Galaxy without the gradients.

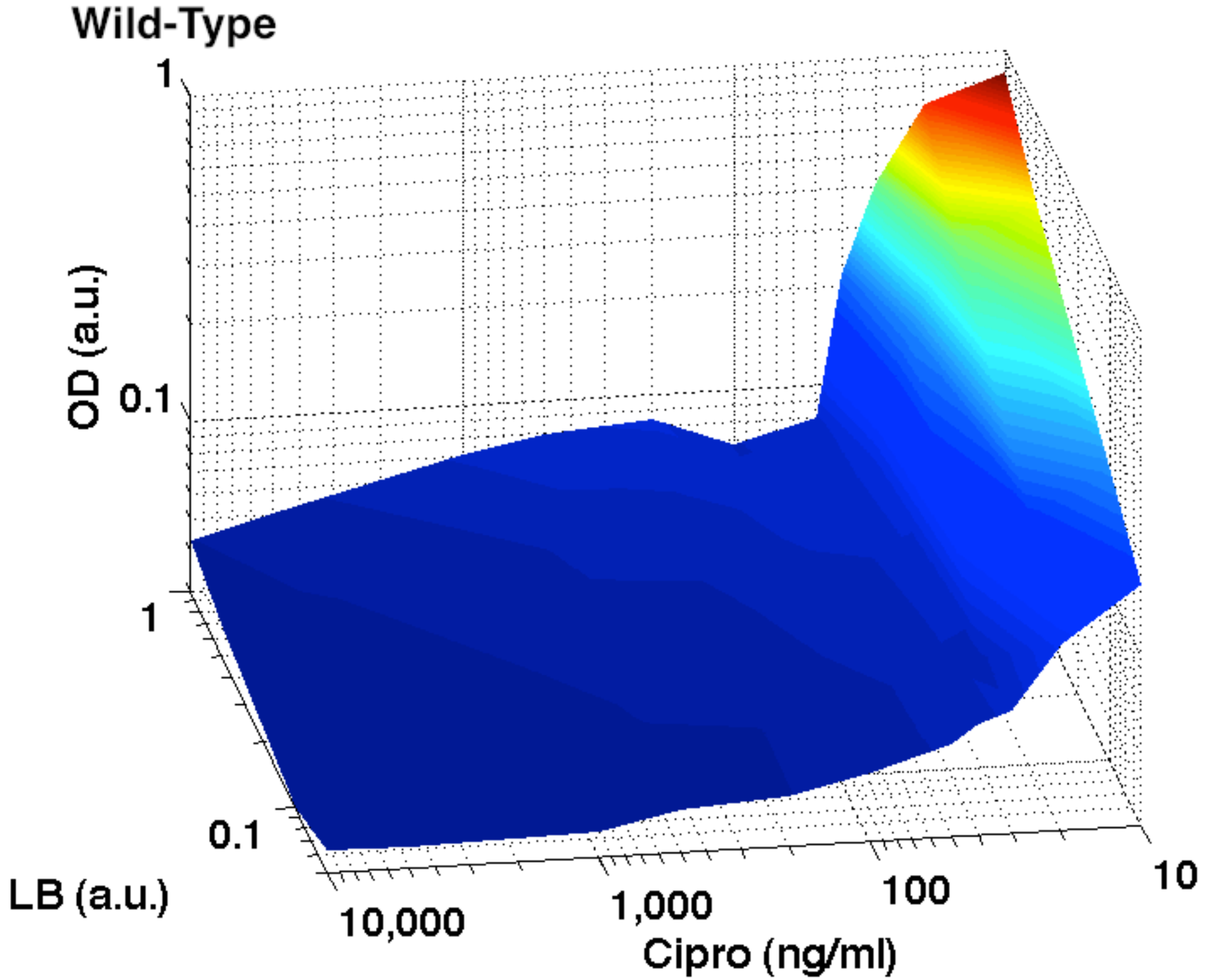


Wild-type: no growth at high Cipro.

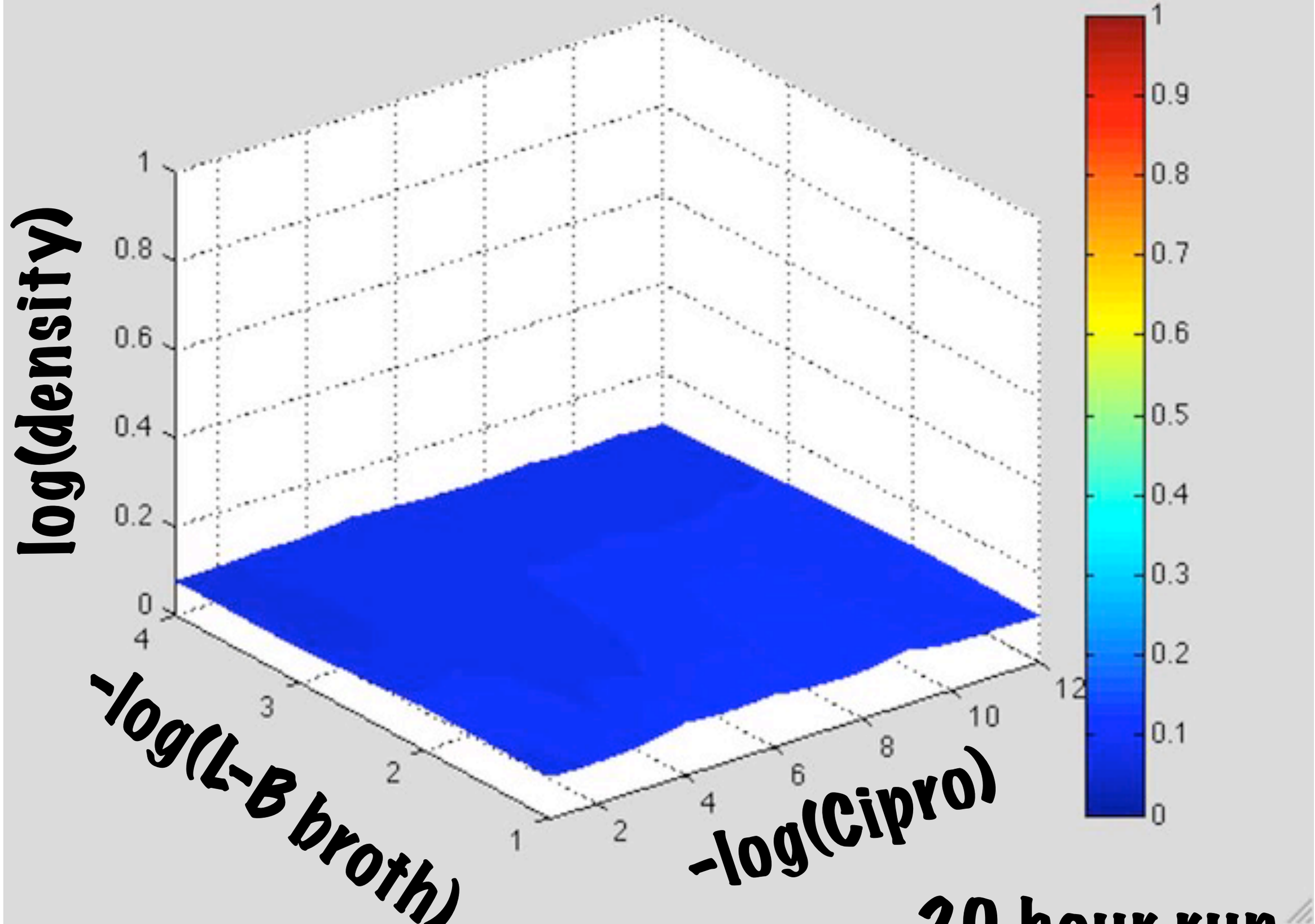


12 hour run

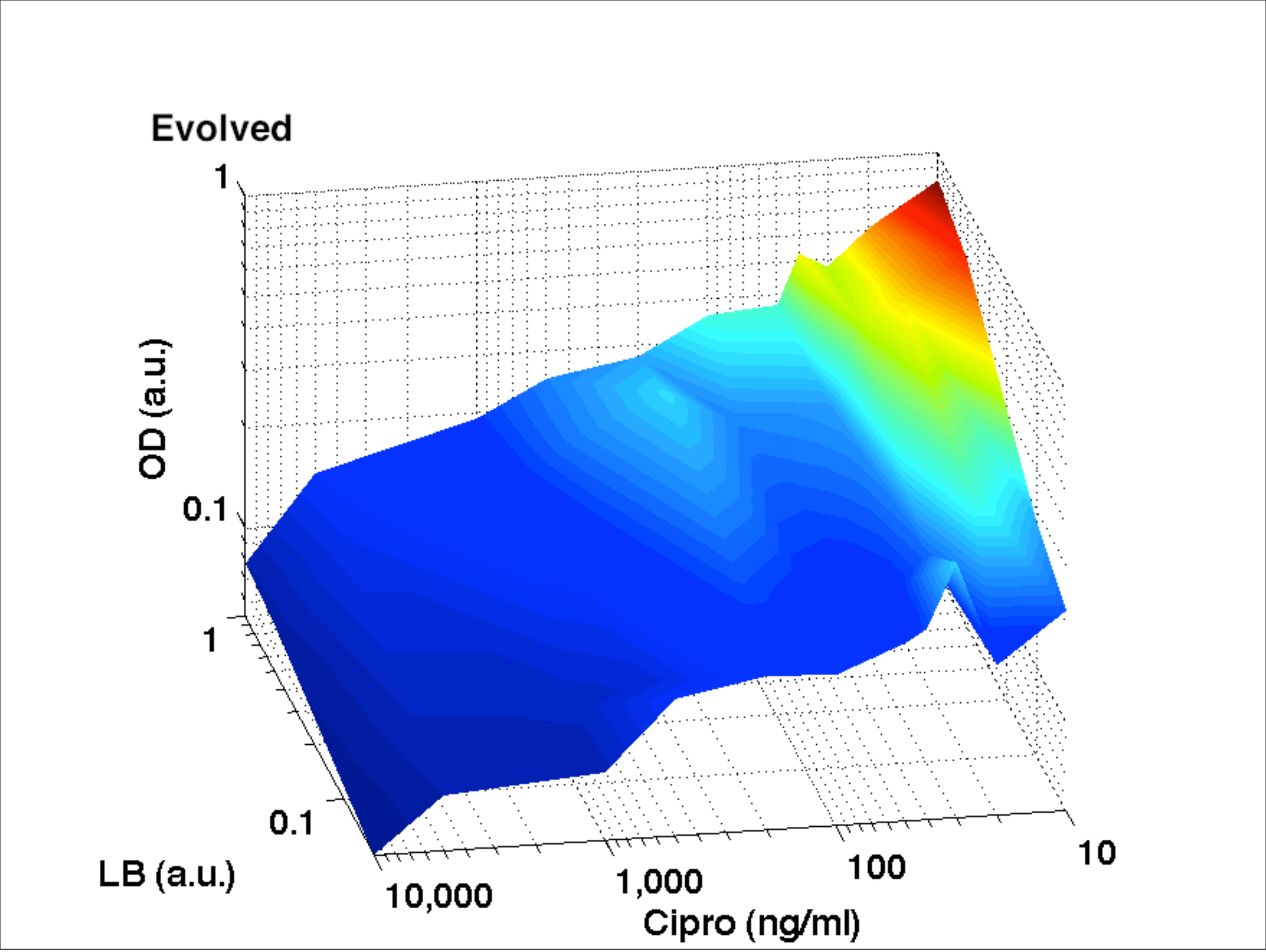
A log-log-log plot of wild-type.

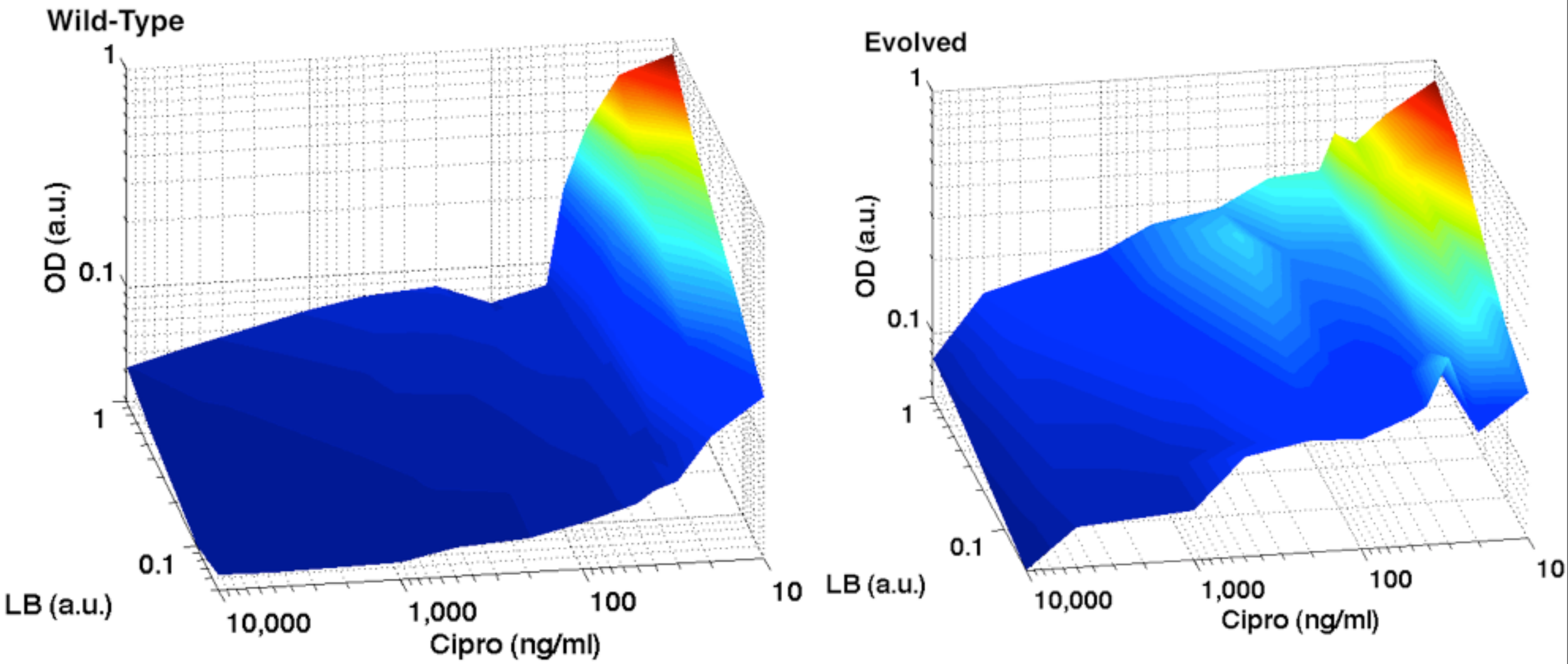


Evolved (post-Death Galaxy)



20 hour run


Evolved
1
OD (a.u.)
0.1
1
0.1
LB (a.u.)
10,000
1,000
100
10
Cipro (ng/ml)



So, I claim this was a smoking gun for evolution and not adaptation, and the inconsequential role of persisters in high stress conditions.

III. Is this true de-novo evolution?

"The results are largely unpublished, for reasons that will become clear; and, frankly, he has excellent technology and great biology, but it was combined with what seems to be a crappy understanding of evolution."

Forgiveness, please. Also, ignore the data.

It should be clear that:

1. We can get rapid (10 hours) emergence of resistance to very high levels of Cipro (x20 MIC).

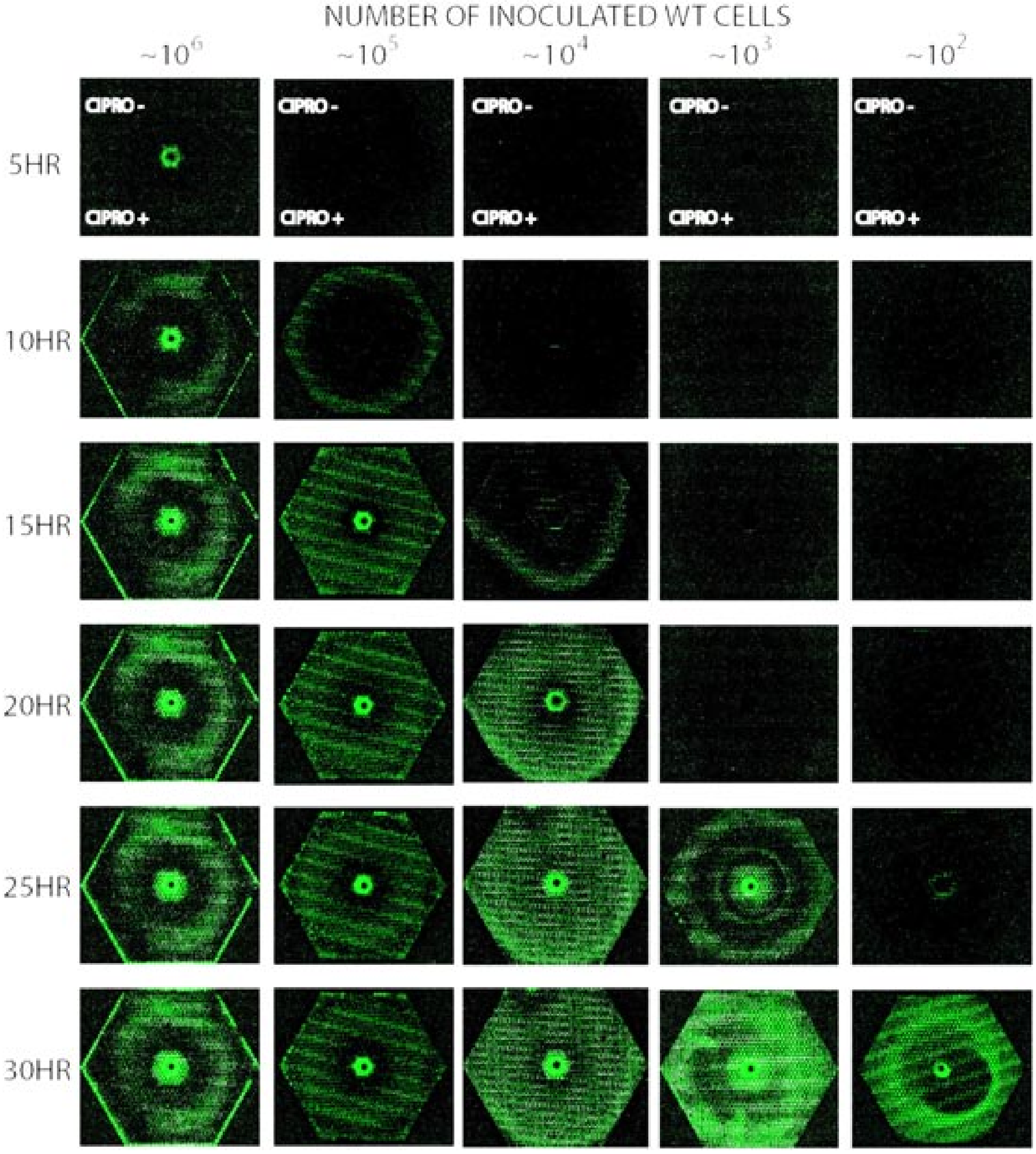
2. You need the Death Galaxy topology: simple "test tubes" don't do it.

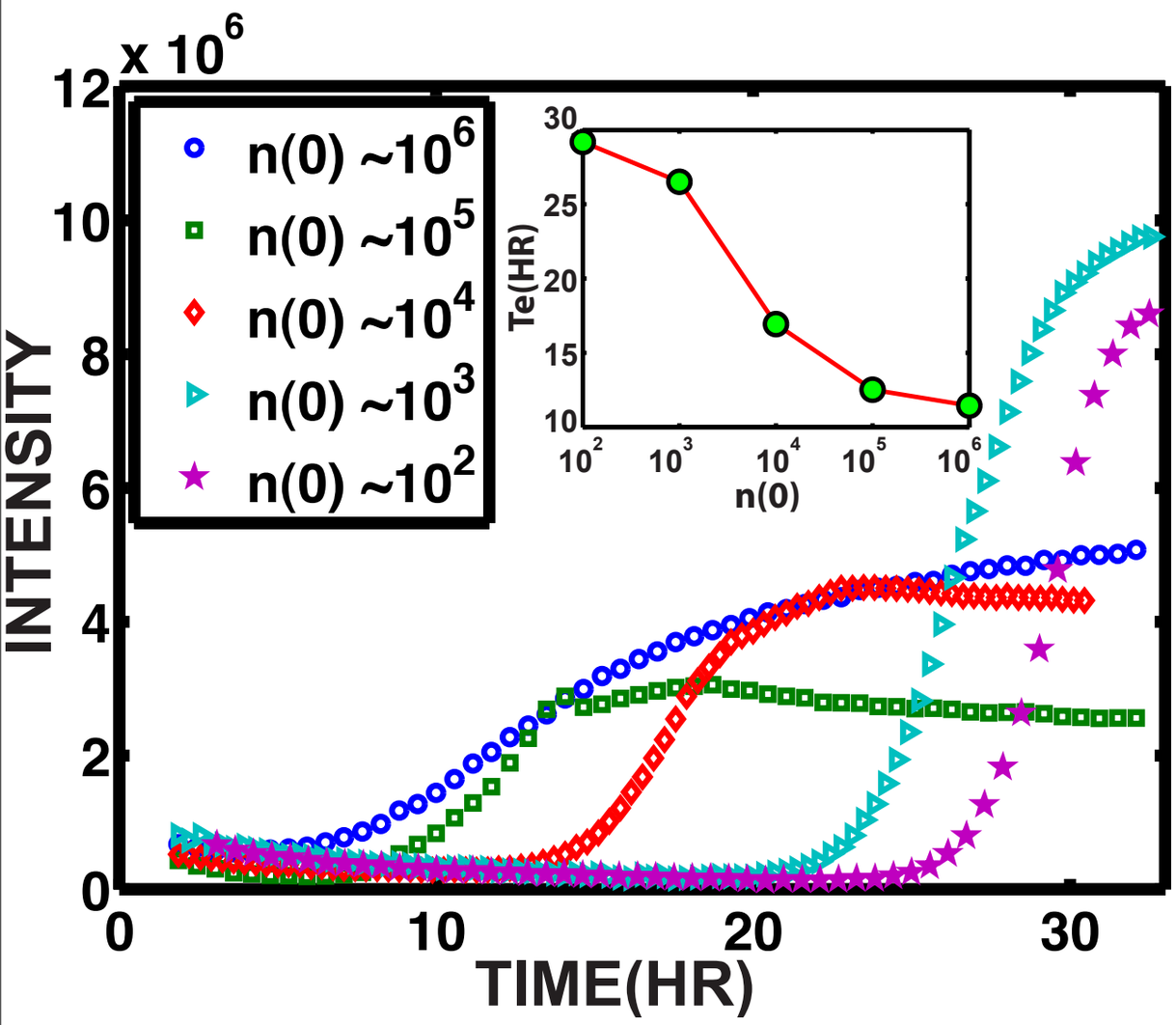
3. Combination of spatial stress gradients AND organismal motility necessary.

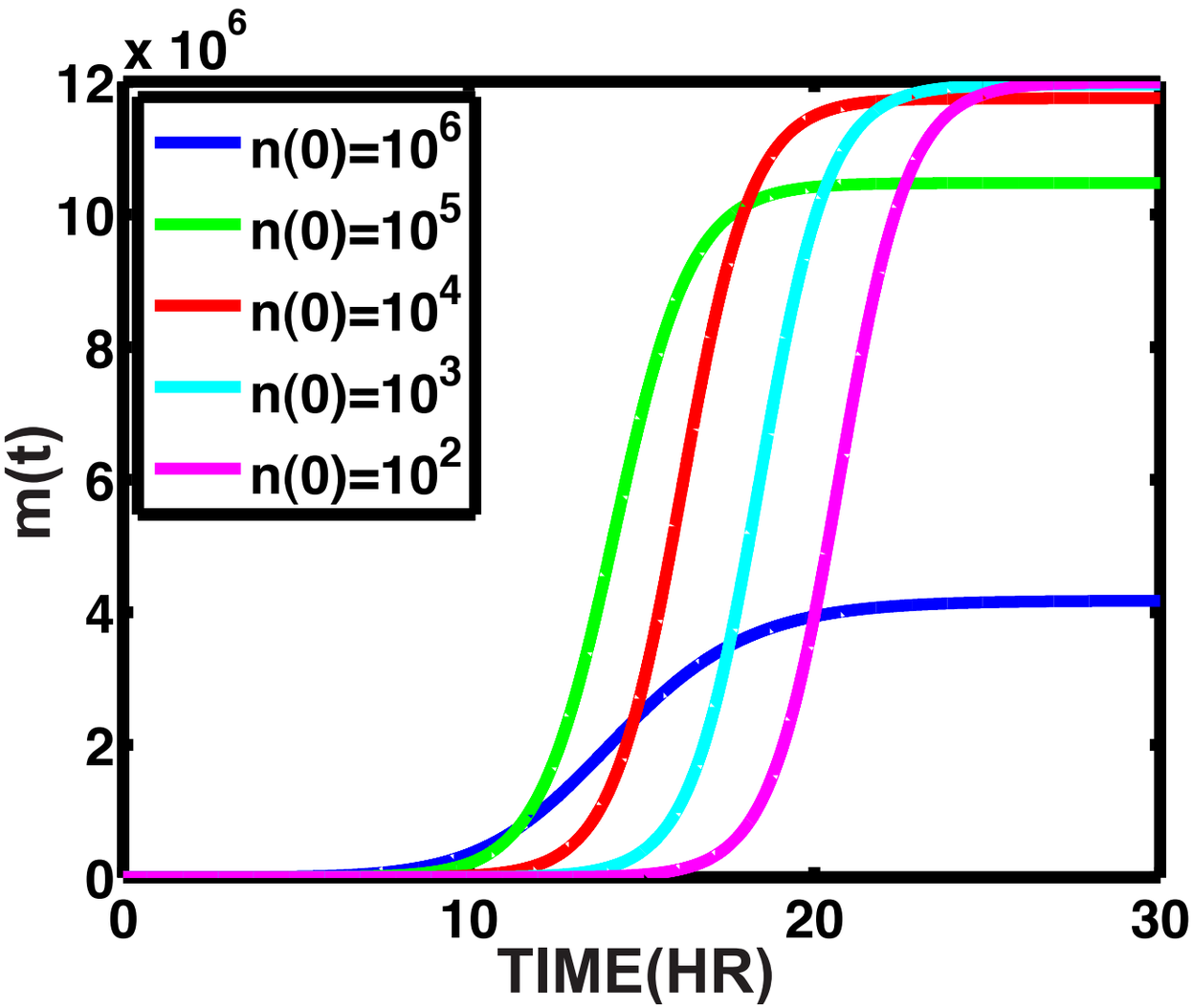
What about population size N? If it is persister, low inoculation will kill it.

What is the sound of 1 E. coli mutating?
100 E.coli inoculation.

What is the sound of 1 E. coli mutating?
100 E.coli inoculation.


NUMBER OF INOCULATED WT CELLS
~10^6
~10^5
~10^4
~10^3
~10^2
5HR
10HR
15HR
20HR
25HR
30HR
CIPRO-
CIPRO+


x 10^6
12
10
8
6
4
2
0
INTENSITY
n(0) ~10^6
n(0) ~10^5
n(0) ~10^4
n(0) ~10^3
n(0) ~10^2
Te(HR)
30
25
20
15
10
10^2
10^3
10^4
10^5
10^6
n(0)
0
10
20
30
TIME(HR)


x 10^6
12
10
8
6
4
2
0
m(t)
n(0)=10^6
n(0)=10^5
n(0)=10^4
n(0)=10^3
n(0)=10^2
0
10
20
30
TIME(HR)

IV. Sequencing: looking behind the phenotype curtain.

“If you look at Austin's sequencing results, they are all over the map.

Shouldn't really have to ask, but... did his experiment have a *control*?”

Sequencing: what exactly is changing in the bacterial genome?

Remember, at this point we look at the end product of the evolution going on, we are at present blind to the spatial and time dynamics occurring within the metapopulations.

2 extreme views:

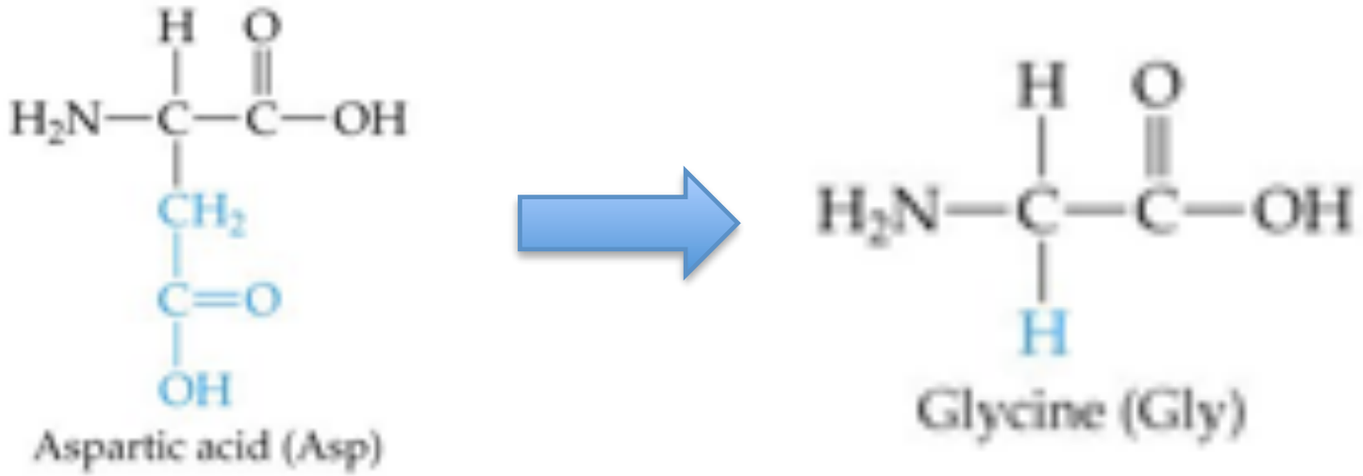
1) Completely random mutations (boring but don't scale).

2) Highly directed mutations (heretic and evil).

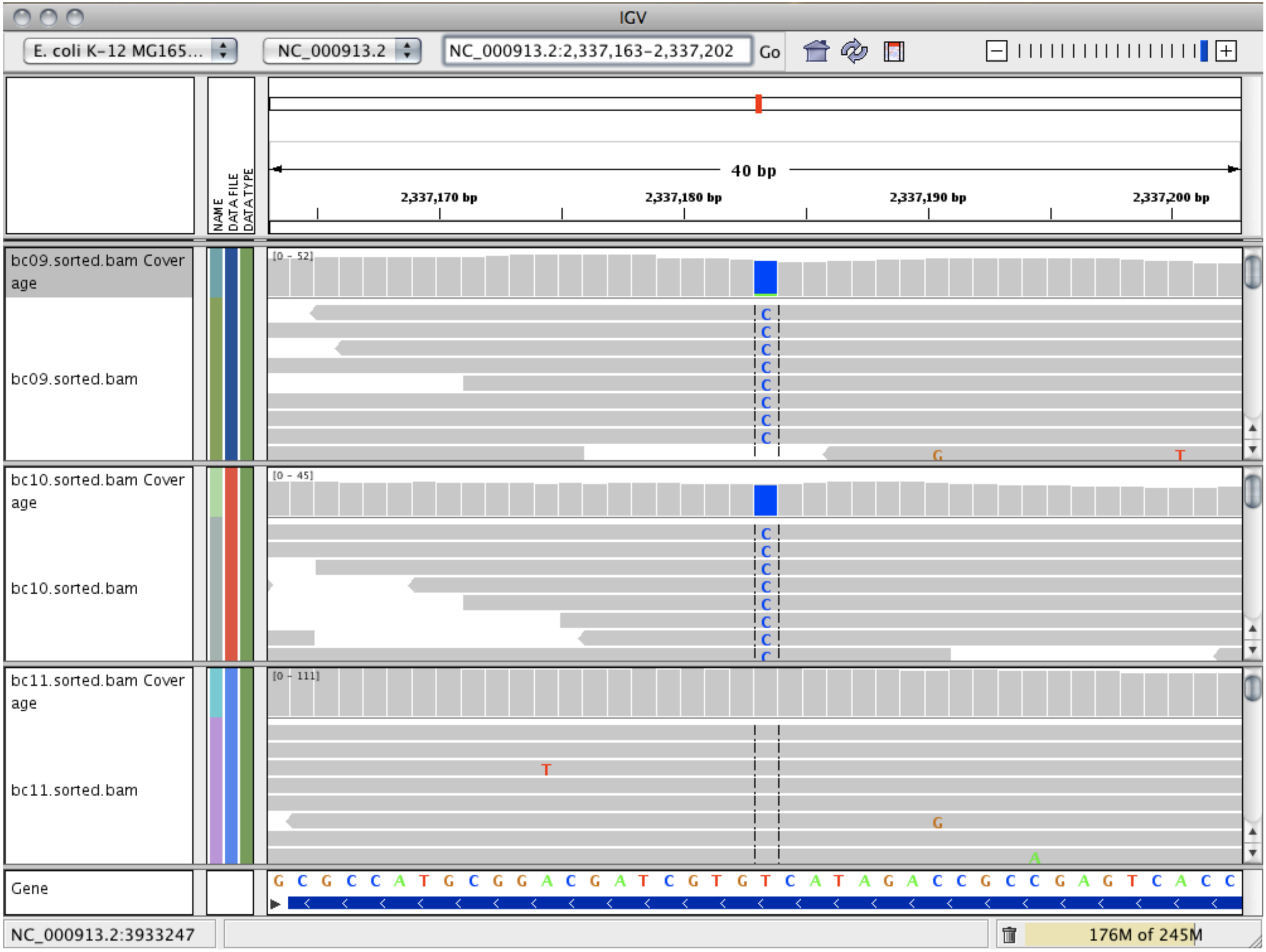
We have found 4 SNPs, "and just 4"

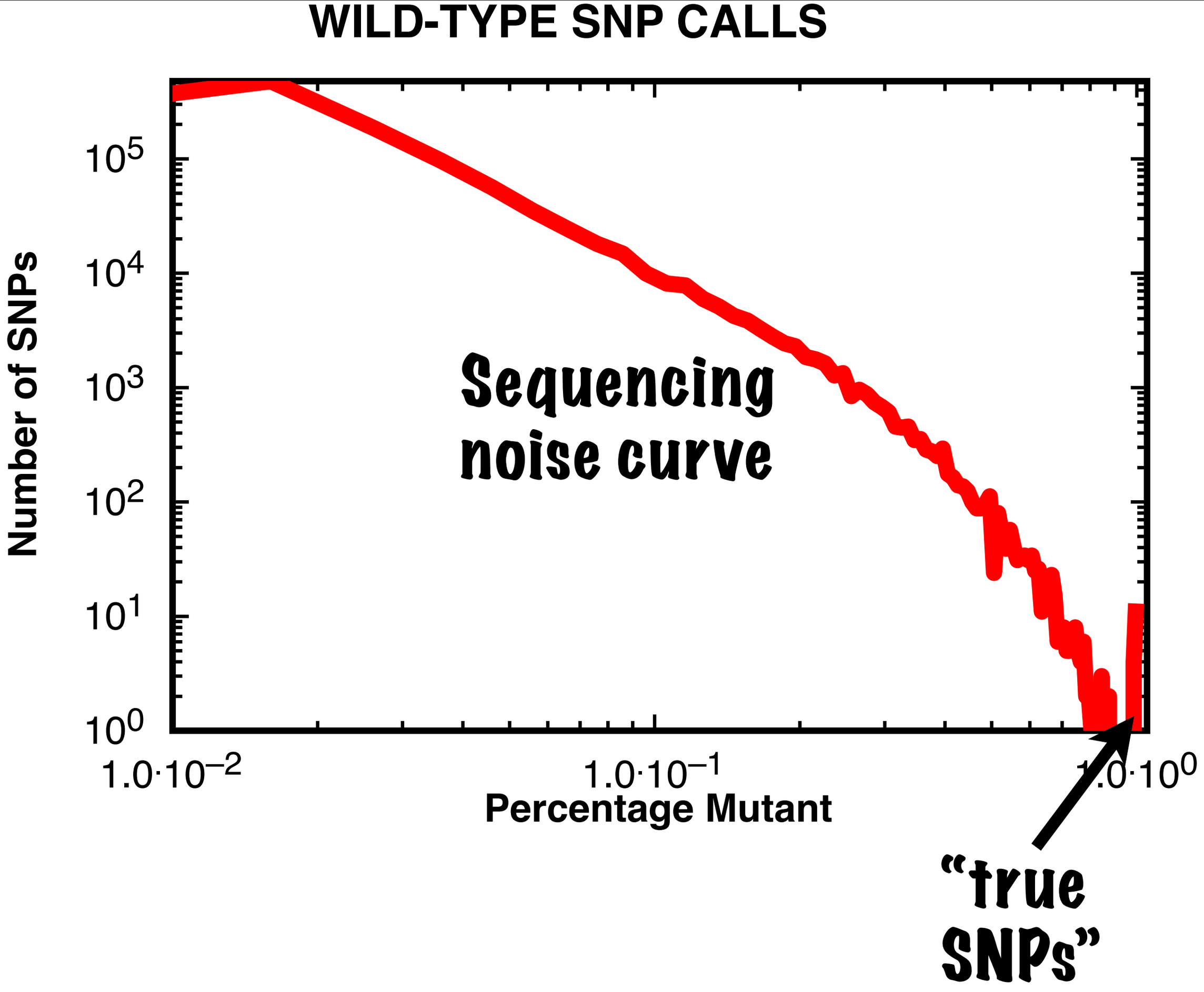
1) Expected this: mutation in gyraseA where Cipro acts. SNP at locus 2,337,183. All samples.

Missense Mutation in gyrA

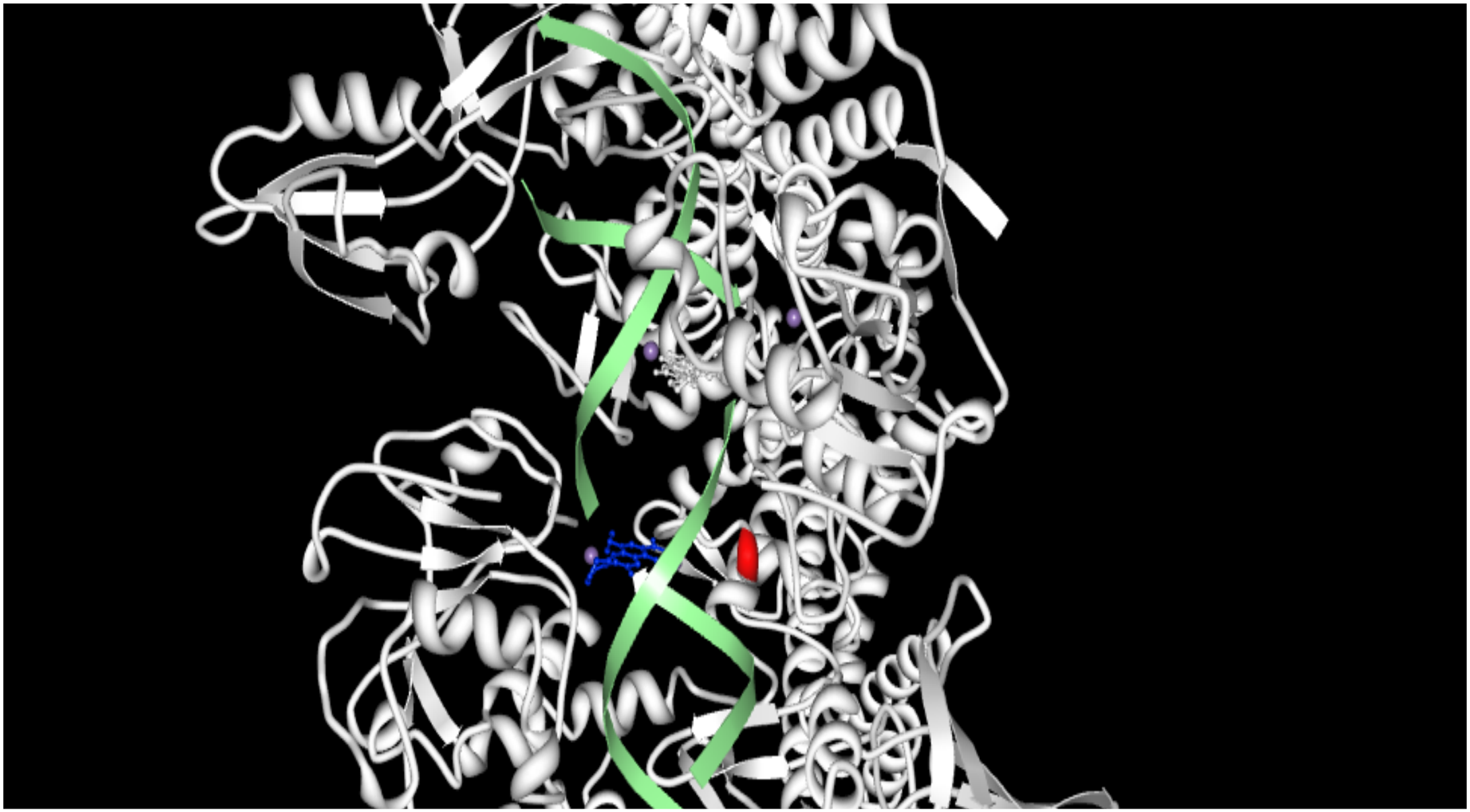


- Mutations in gyrA have been shown to impart cipro-resistance to e. coli
- Previous studies show a D87N also imparts resistance
- Mutations that impart resistance most often occur in active binding site


IGV
E. coli K-12 MG165...
NC_000913.2
NC_000913.2:2,337,163-2,337,202
Go
NAME
DATA FILE
DATA TYPE
40 bp
2,337,170 bp
2,337,180 bp
2,337,190 bp
2,337,200 bp
bc09.sorted.bam Coverage
[0 - 52]
bc09.sorted.bam
bc10.sorted.bam Coverage
[0 - 45]
bc10.sorted.bam
bc11.sorted.bam Coverage
[0 - 111]
bc11.sorted.bam
Gene
G C G C C A T G C G G A C G A T C G T G T C A T A G A C C G C C G A G T C A C C
NC_000913.2:3933247
176M of 245M


WILD-TYPE SNP CALLS
Number of SNPs
10^5
10^4
10^3
10^2
10^1
10^0
$1.0 \cdot 10^{-2}$
$1.0 \cdot 10^{-1}$
$1.0 \cdot 10^{0}$
Percentage Mutant
Sequencing noise curve
"true SNPs"

Gyrase (white), dsDNA (green), cipro (blue), mutated base (red)



We found another SNP in an unexpected place: pumps that remove toxins. Should have expected it. One SNP does not a phenotype make.

Mutation in gyrA alone is not enough to impart resistance

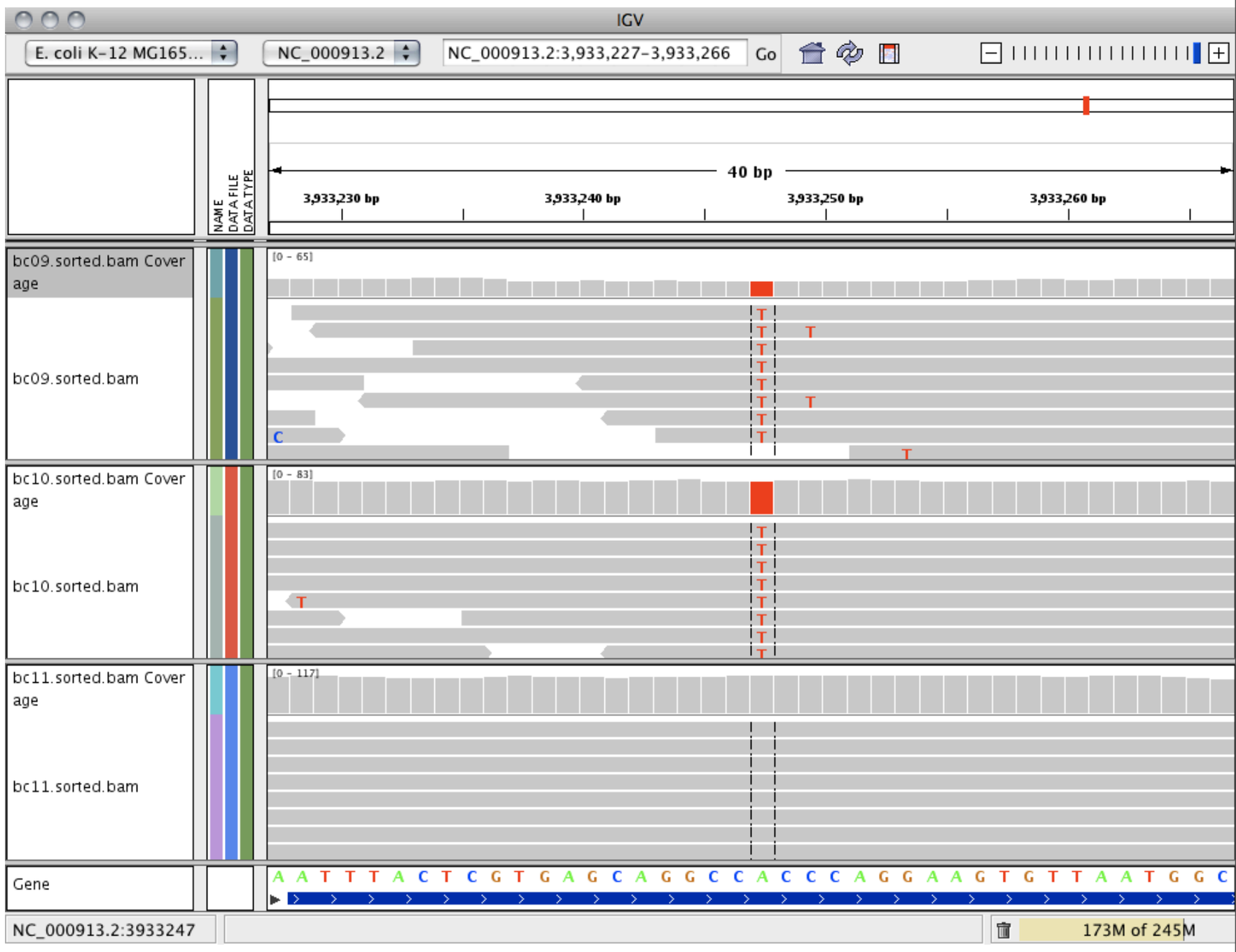
Table 1. Correlation between fluoroquinolone (ciprofloxacin/levofloxacin) susceptibility of 59 *Pseudomonas aeruginosa* clinical isolates and mutations in *gyrA* and *mexR* genes.

Fluoroquinolone susceptibility (no. of isolates)	Mutations in *gyrA* only	Mutations in *mexR* only	Mutations in both *gyrA* and *mexR*	No mutations in *gyrA* or *mexR*
Resistant (12)	4	0	4	4
Intermediate (6)	1	1	1	3
Susceptible (41)	1	12	1	27

- Correlation between resistance and a point mutation in gyrA is not 100%
- Resistant strains may not have a mutation in gyrA at all

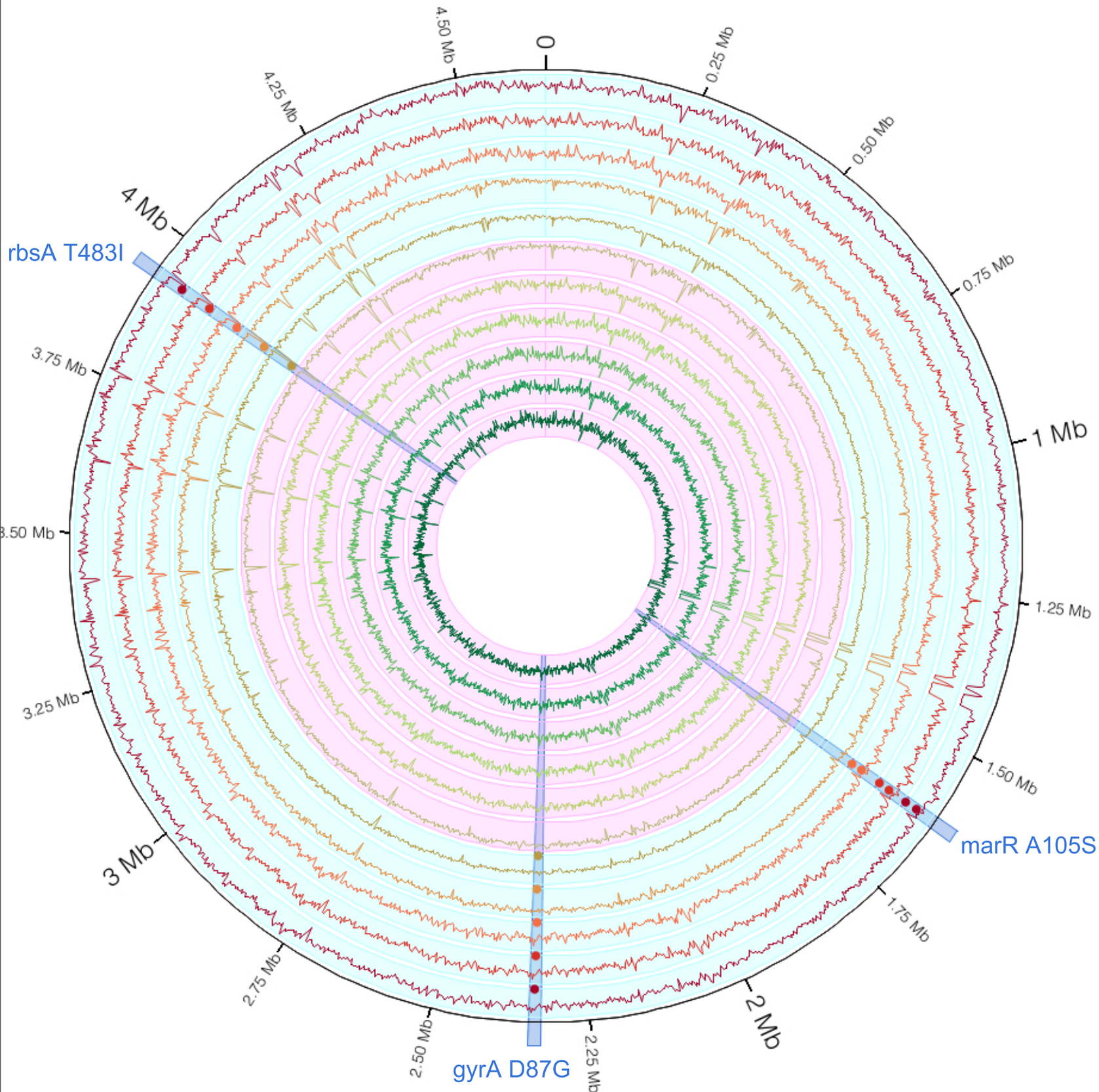
Gorgani, N. (2009). Detection of point mutations associated with antibiotic resistance in Pseudomonas aeruginosa. International journal of antimicrobial agents, 34(5), 414-.

A missense A to T in base 3,933,247 in a region coding for the rbsA gene which is a component of the ribose ABC transporter complex and been previously reported to export other antibiotics (Erythromycin, Tylosin, and Macrolide).



Also found 2 SNPs (1,617, 461: A to C and 1,617, 460:C to G) in the marR operon, which is the equivalent of an oncogene for bacteria:

The mar regulon identified in Escherichia coli (mar-Eco) plays a key role in the expression of a multidrug resistance phenotype, and specific mutations located in marR have been identified in resistant strains. The regulatory function associated with the marA locus simultaneously induces a decrease in antibiotic uptake by altering the porin content of the outer membrane and an increase of antibiotic ejection by activating efflux mechanisms. This response supports an efficient resistance to a range of commonly used antibiotics.

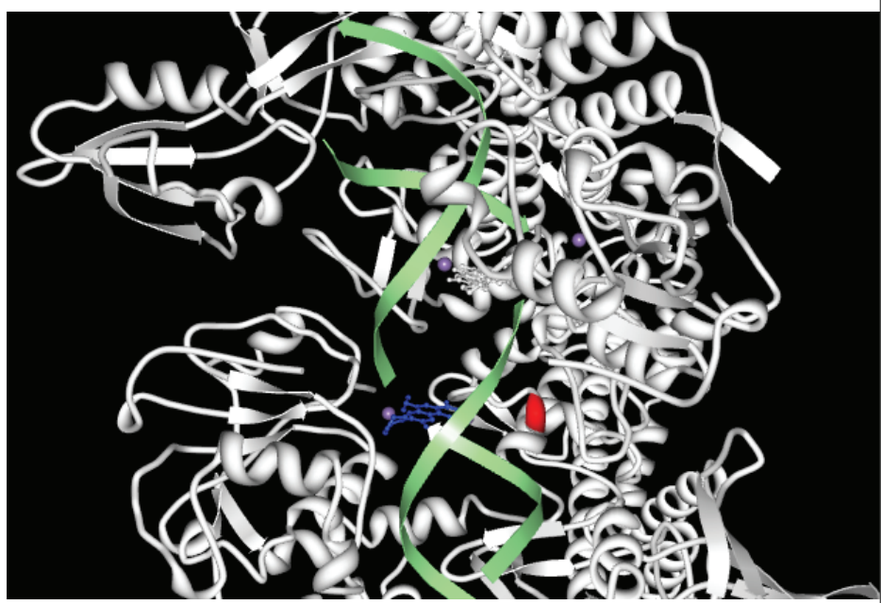


Ciprofloxacin Resistant
DG-2a
DG-2b
DG-3
DG-1a
DG-1b

Ciprofloxacin Susceptible
WT_a
WT_b
DG-4a
DG-4b
Plate
DG-4c

SNPs

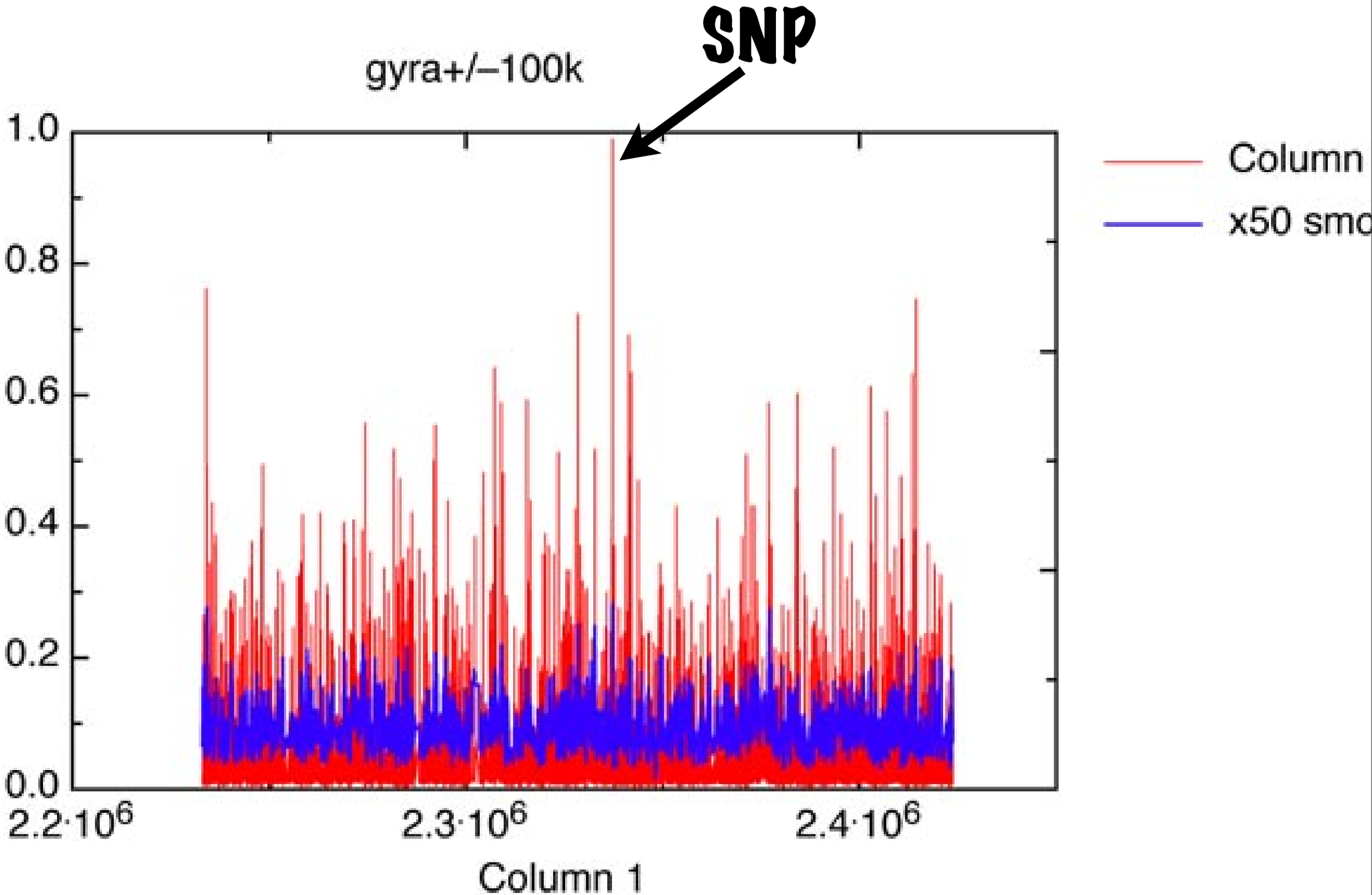
Feature	Position	Mutation
gyrA	2,337,183	D87G
rbsA	3,933,247	T483I
marR	1,617,460	A105S
marR	1,617,461	A105S



There is a problem. Where are the passenger mutations? We see 4 clear SNPs in functional places and nothing else.

If this came from random mutations, there should be lots of neutrals. There aren't any.

But directed mutations in the area of gyrA, although heretic and no doubt evil, don't seem to be there either. We see single spikes in the mutation landscape, and that is "troubling"


SNP
gyra+/−100k
1.0
0.8
0.6
0.4
0.2
0.0
2.2·10^6
2.3·10^6
2.4·10^6
Column 1
Column
x50 smo

Personally, I find this pretty shocking:

Not only are we finding rapid emergence of antibiotic resistance in bacteria scaling down to very small numbers of bacteria, but also we see rapid and innovative finding of ways to bypass the antibiotics.

These mutations occur rapidly and in highly specific places that are highly functional.

I think the system knows what it is doing, and Haldane had the right idea: things are queerer than we think

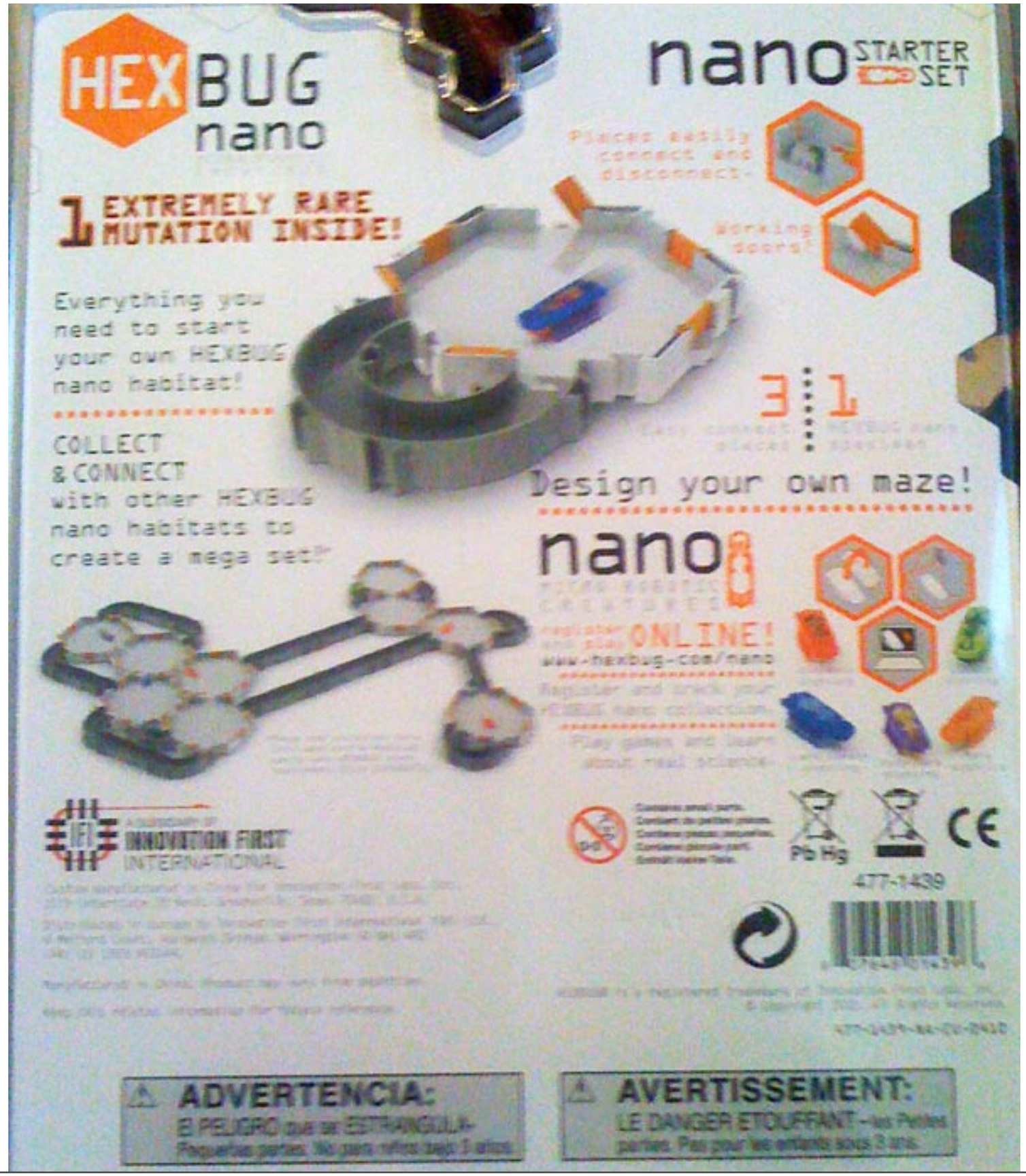
My Dark Thoughts in the Night are:

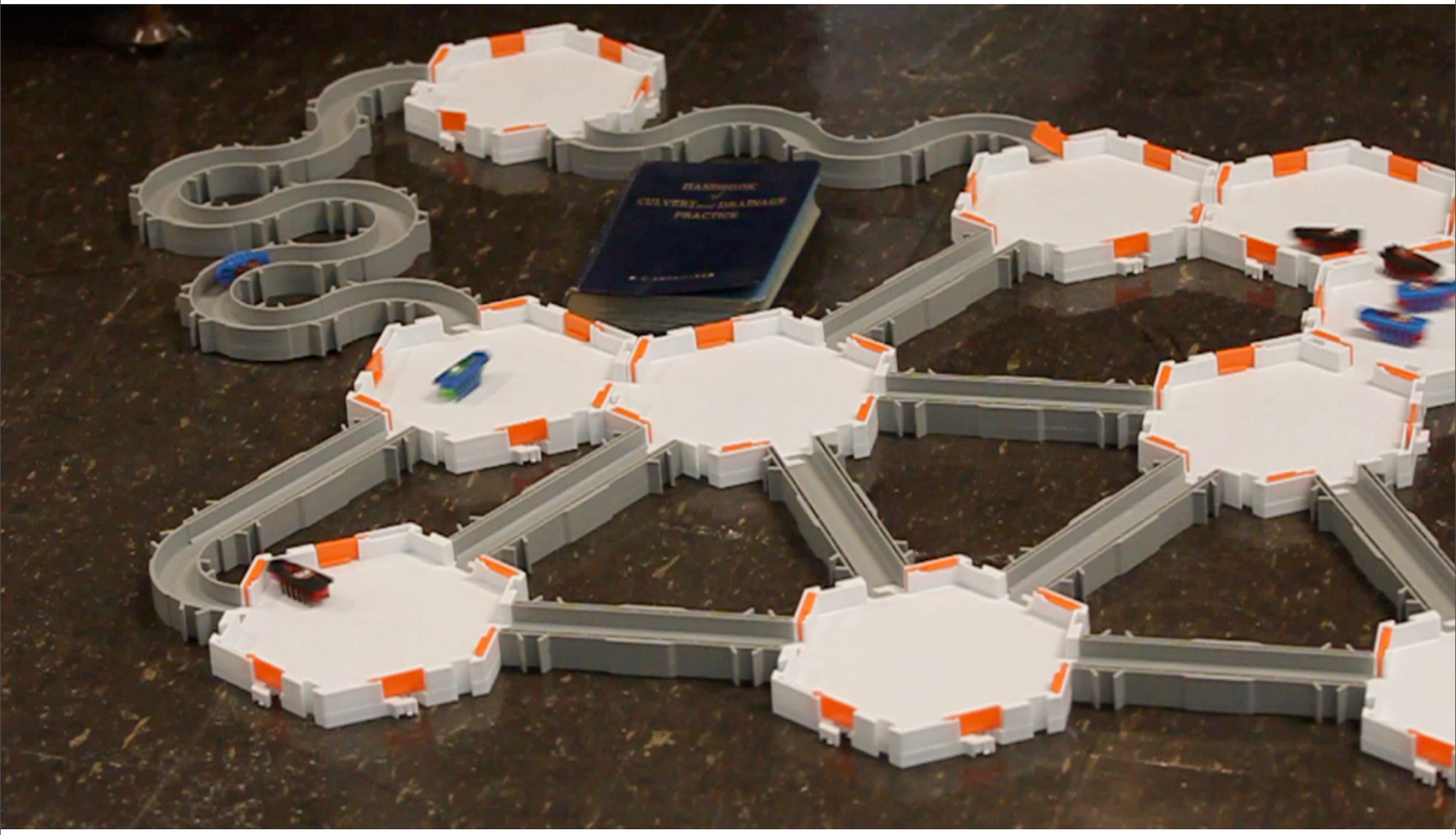
1) Cancer is not a disease. Evolution requires the deliberate generation of genomic diversity because random mutations are too slow. Cancer is the "tail end" of a mechanism in place because it provides fitness advantage to the species, not you.

2) Continued attempts to destroy cancer will only result in even more rapid evolution under stress. We are doing the wrong thing.

V. Toy Story

The Chinese have stolen my idea!





Thanks for all the fish!

http://arstechnica.com/science/news/2010/12/a-tale-of-an-ingenious-experiment-with-a-dubious-interpretation.ars

I could say something trite about the hazards of letting physicists do biology. But the point is that Austin's experiments are ingenious, beautiful, and useful. Unfortunately, he has gotten totally out of his depth in interpreting them and would benefit by bringing an experienced evolutionary biologist (along with an experienced cell biologist) into his team to help interpret the results. Until he does so, he will remain on the margins.