Borrelia Biofilm: Is it real, and why is it important in chronic Lyme disease?

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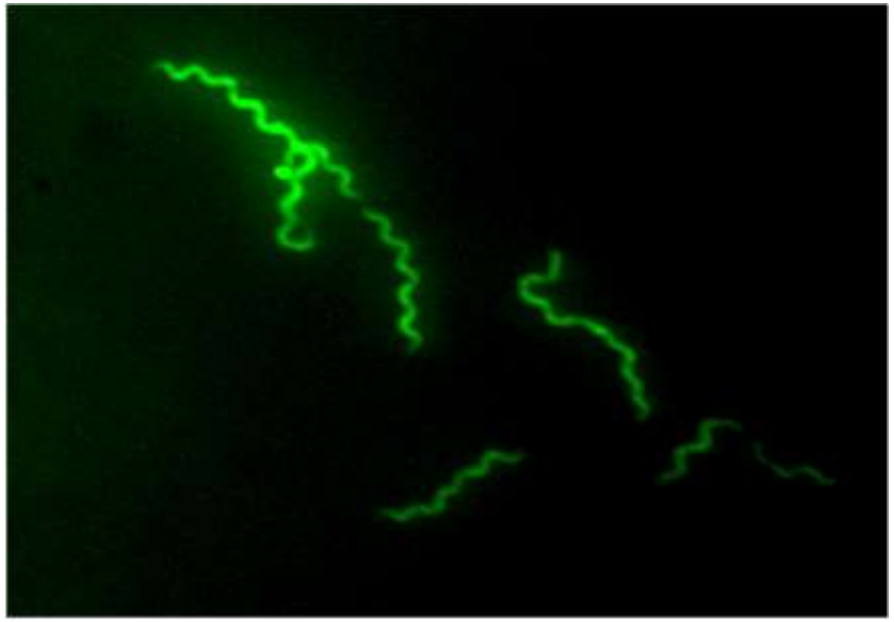
Borrelia burgdorferi the spirochete that causes Lyme disease

In 1982, the etiologic agent of Lyme disease was discovered by Dr. Willy Burgdorfer who isolated spirochetes belonging to the genus *Borrelia* from the mid-guts of *Ixodes* ticks.

He showed that these spirochetes reacted with immune serum from patients that had been diagnosed with Lyme disease. Subsequently, the etiologic agent was given the name *Borrelia burgdorferi.*



Borrelia burgdorferi – University of New Haven

How long did we know about Lyme disease?

John Josselyn, who visited New England in 1638 and again from 1663–1670, wrote

"there be infinite numbers of tikes hanging upon the bushes in summer time that will cleave to man's garments and creep into his breeches eating themselves in a short time into the very flesh of a man. I have seen the stockings of those that have gone through the woods covered with them"

The examination of preserved museum specimens has found *Borrelia* DNA in an infected *Ixodes ricinus* tick from Germany that dates back to 1884, and from an infected mouse from Cape Cod that died in 1894.

The 2010 autopsy of Ötzi the Iceman, a 5,300-year-old

mummy, revealed the presence of the DNA sequence of

Borrelia making him the earliest known human with Lyme disease.

Google Image Library

So how can we eliminate *Borrelia*?

In vitro studies for different species of *Borrelia* – Minimum bactericidal concentrations (microgram/ml)

Doxycycline:	0.25-**25.0**
Penicillin	0.15-**6.5**
Azitromycin:	0.015-**2.0**
Erythromycin	0.06->0.5
Clarithromycin	0.06-0.5
Telitromycin	0.002-0.03
Amoxicillin:	0.4-**8.00**
Ceftriaxone:	0.03-2.00*
Ciproflaxin	0.5-**16.0**
Tigecycline	0.05-0.19

- *Russel et al 1987, Agger et al 1992; Dever et al 1992, Levin at all 1993; Sicklinger et al 2003, Hunfeld et al 2004, 2005*, Kim et al 2006, Yang et al 2009, Brorson et al 2009*

In vitro and *clinical* data – do they agree?

"Survival of *Borrelia burgdorferi* *in patients* with Lyme borreliosis *treated with antibiotics* "
Preac-Mursic V et al 1989

"*In vitro* results have no proven correlation with antimicrobial clinical effectiveness *in vivo*"
Moody KD et al 1994

"Culture positive and PCR positive blood after antibiotics therapy"
Oksi J et al 1999

"Clinically treatment failures occur in 5 to 10% of EM patients (oral doxycycline or amoxicillin for 14 to 30 days)"
Smith RP et al 2002

But how about the *in vivo* studies?

Treatment with tetracycline, erythromycin or doxycycline in mice failed to eradicate acute *Borrelia* infection. *Moody KD et al 1994*

Chloramphenicol and azithromycin failed to eradicate the organism - *Moody KD et al 1994*

In a dog model, results showed that antibiotic-treated dogs (doxycycline and amoxicilllin, 30 days) continued to have persistent *Borrelia*-specific DNA in their tissue albeit at lower levels than observed in untreated animals. *Straubinger RK et al 1997*

But how about Ceftriaxone (Rocephin)?

Bockenstedt LK, Mao J, Hodzic E et al. Detection of attenuated, noninfectious spirochetes in *Borrelia burgdorferi*-infected mice after antibiotic treatment. J Infect Dis 2002; 186: 1430–7.

Hodzic E, Feng S, Holden K et al. Persistence of *Borrelia burgdorferi* following antibiotic treatment in mice. Antimicrob Agents Chemother 2008; 52: 1728–36.

SUMMARY: **A low numbers of noncultivable spirochetes**, detected by PCR following antibiotic treatment which can be **acquired by ticks**, transmitted by ticks, survive the molts between larvae to nymphs to adults, infect mice, and **form of morphologically identifiable spirochetes**

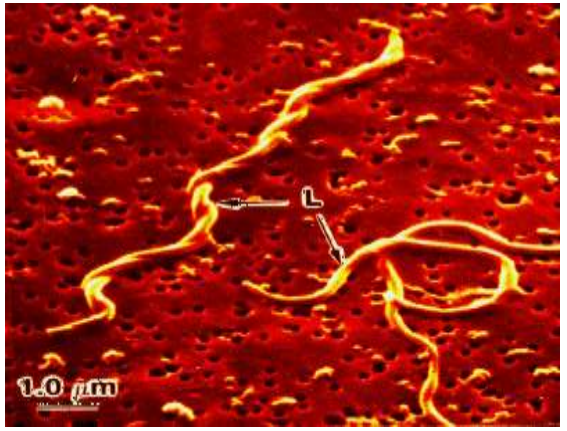
The different forms of Borrelia

Borrelia burgdorferi can convert between cyst, non-motile and normal motile spirochete forms.

The cystic forms are resistant to most antibiotic treatments and difficult to detect in the body.

- http://www.lymeinfo.net/medical/LDAdverseConditions.pdf

B. burgdorferi after exposure to penicillin concentration of 0.125 mg/l. Coiled up spirochete forming a spherical structure (spheroplast).
Schaller M; Neubert U. 1994



New York State Department of Health

The most recognized forms of *Borrelia burgdorferi*

- Spirochetes
- Round bodies (cysts, granules)
- L-forms
- Biofilm

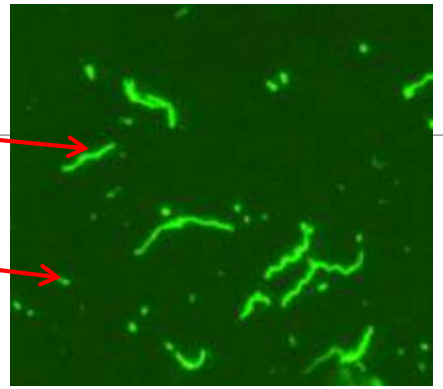


Photo by Namrata Pabbati MS

Photo by Alan MacDonald MD, 2006

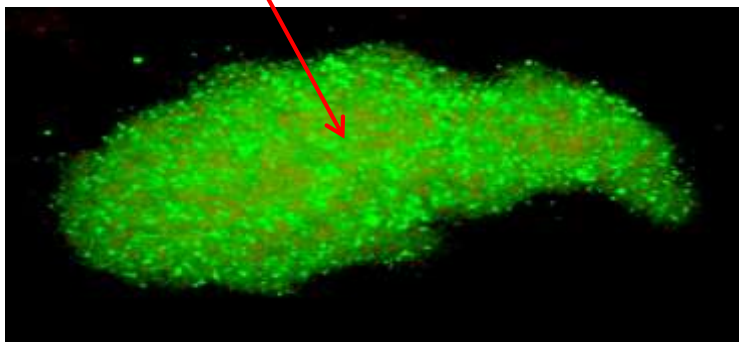


Photo by David Luecke MS

Round bodies *in vivo*

Neocortical Borreliosis and Alzheimer's Disease Demonstration of a Spirochetal Cyst Form

MacDonald A 1988

- **An unexpected observation was the identification of cystic forms of the *Borrelia* spirochete by dark field microscopy cultured hippocampus**

Conversion of Borrelia garinii Cystic Forms to Motile Spirochetes *In Vivo*.

Gruntar I et al 2001

- ***Borrelia garinii* cystic forms maintain their capability to reconvert into normal spirochetes not only *in vitro* but also *in vivo* and can therefore be considered infective, at least in BALB/c mice.**

Agents for the cystic forms (RB)

In Vitro Study of the Susceptibility of Mobile and Cystic Forms of Borrelia burgdorferi to Metronidazole *Brorson O et al 1999*

"*B. burgdorferi has the ability to make cystic forms both in vivo and in vitro, e.g. when exposed to* antibiotics commonly used for treating Lyme borreliosis. This phenomenon, combined with the ability of the **cysts to reconvert to normal mobile spirochetes** may explain a reactivation of the disease– and not a "post Lyme syndrome" as postulated by other researchers."

Additional Brorson O et al *in vitro* studies for the antibiotic sensitivity of the cystic (round bodies) form

2001 Susceptibility of motile and cystic forms of *Borrelia burgdorferi* to **ranitidine bismuth citrate**. Int Microbiol, 4(4):209-15.

2002 An *in vitro* study of the susceptibility of mobile and cystic forms of *Borrelia burgdorferi* to **hydroxychloroquine** Int Microbiol, 5(1):25-31.

2004 An *in vitro* study of the susceptibility of mobile and cystic forms of *Borrelia burgdorferi* to **tinidazole**. Int Microbiol, 7(2):139-40.

2009 Destruction of spirochete *Borrelia burgdorferi* round-bodies by **tigecycline** PNAS 106(44):18656-61.

Ineffectiveness of Tigecycline against Persistent *Borrelia burgdorferi in vivo*

Non-cultivatable *Borrelia burgdorferi* could be isolated from mice treated with ceftriaxone and tigecycline.

Mice remained **infected with non-dividing, but infectious spirochetes**, particularly when antibiotic treatment was commenced during the chronic stage of infection

Barthold SW et al 2010

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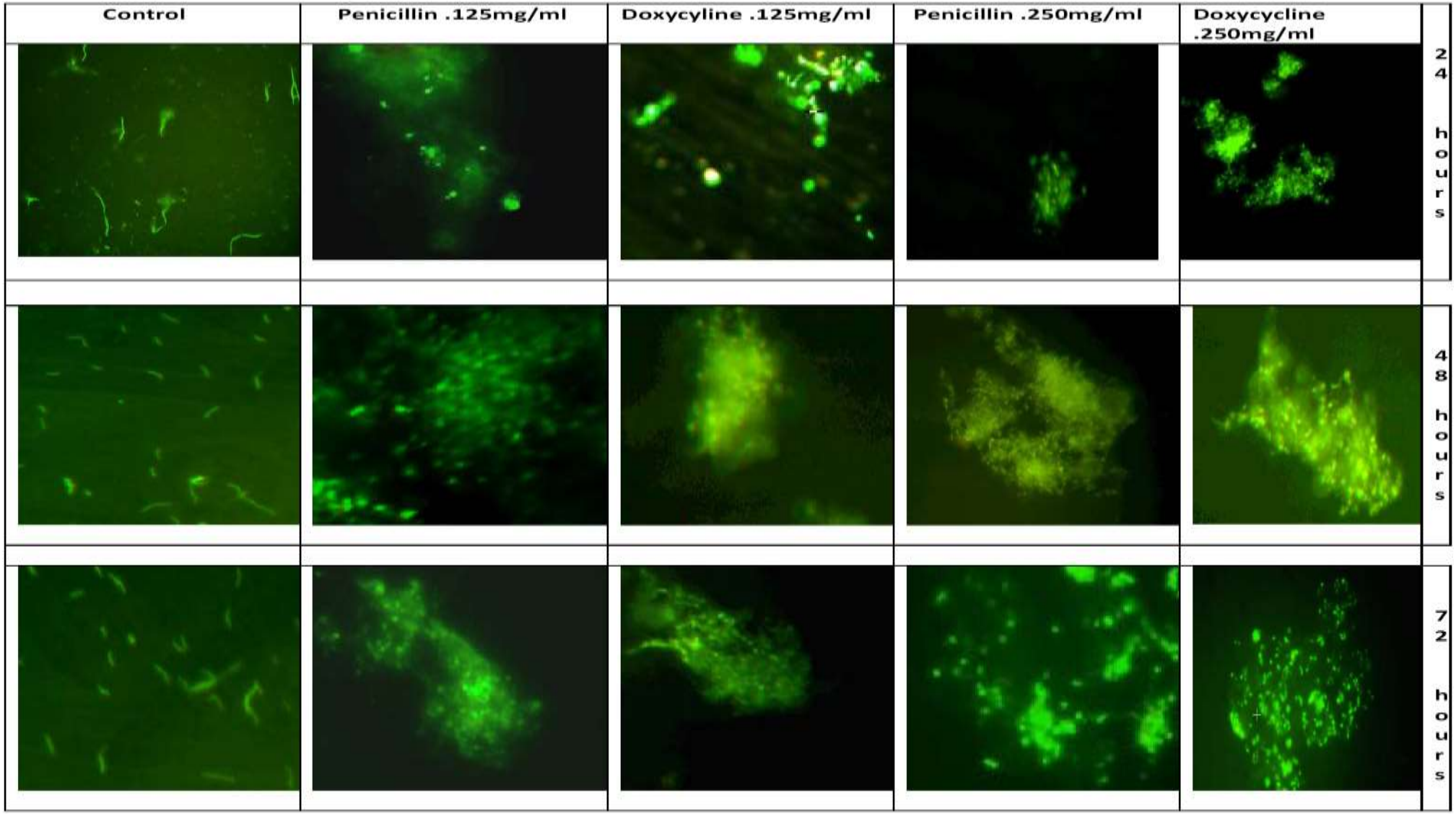
Embers ME et al:
Persistence of *Borrelia burgdorferi* in Rhesus Macaques following Antibiotic Treatment of Disseminated Infection. PLoS ONE 2012

- found that Borrelia persist after 90 days in monkeys treated for chronic Lyme disease.

- the antibody tests used to diagnose Lyme disease fail to detect disease in late Lyme disease at least 50% of the time.

So what can we do now ????

What other escape route Borrelia could have??



Rao P, Azano, D & Sapi E, unpublished data 2008

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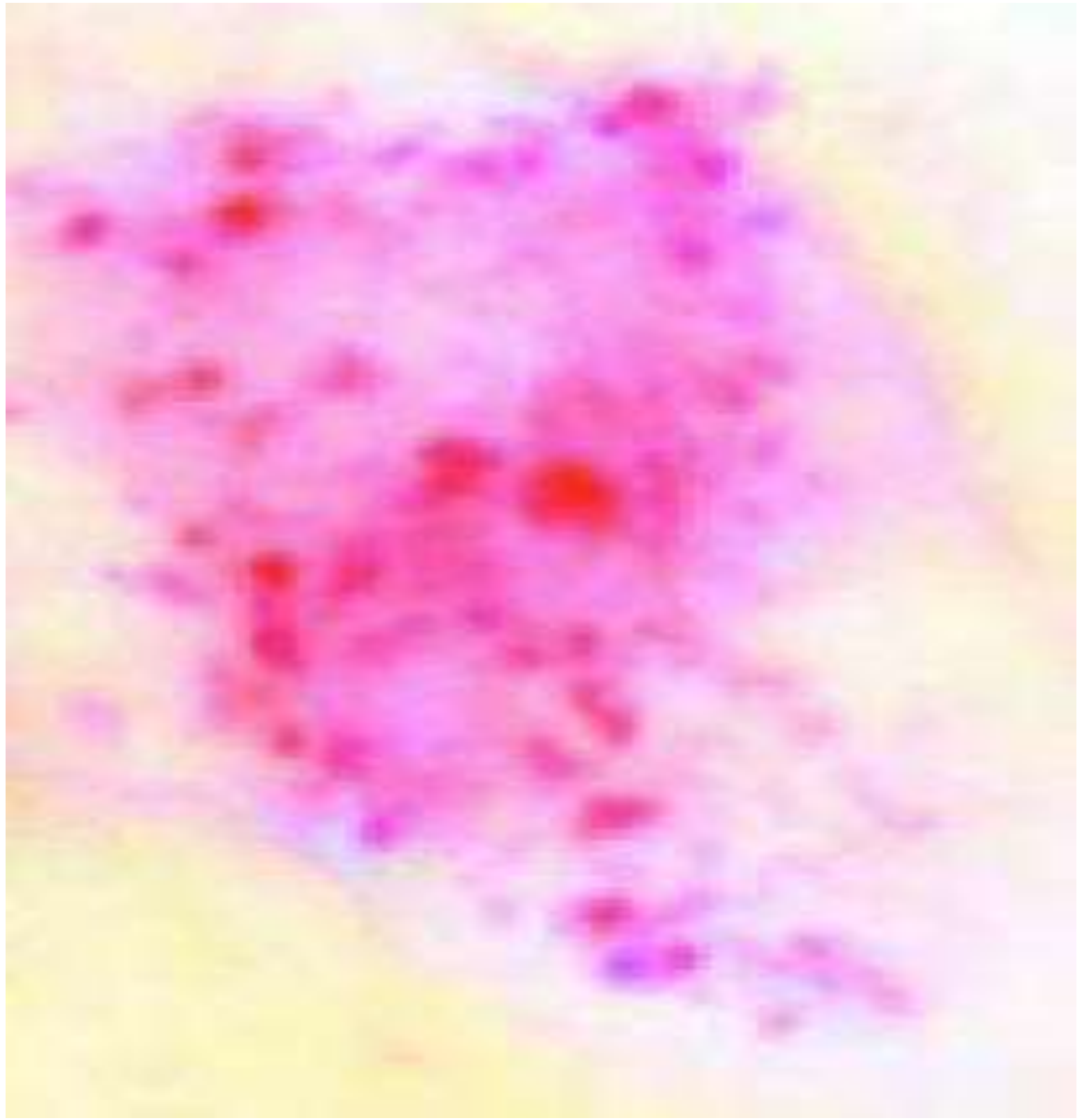

K. Eisendle et al. AJCP 2007,127:213-222 Acrodermatitis Chronica Atrophicans Immunohistochemistry

“Granular forms of *Borrelia burgdorferi* in a “colony” with a “reddish veil”

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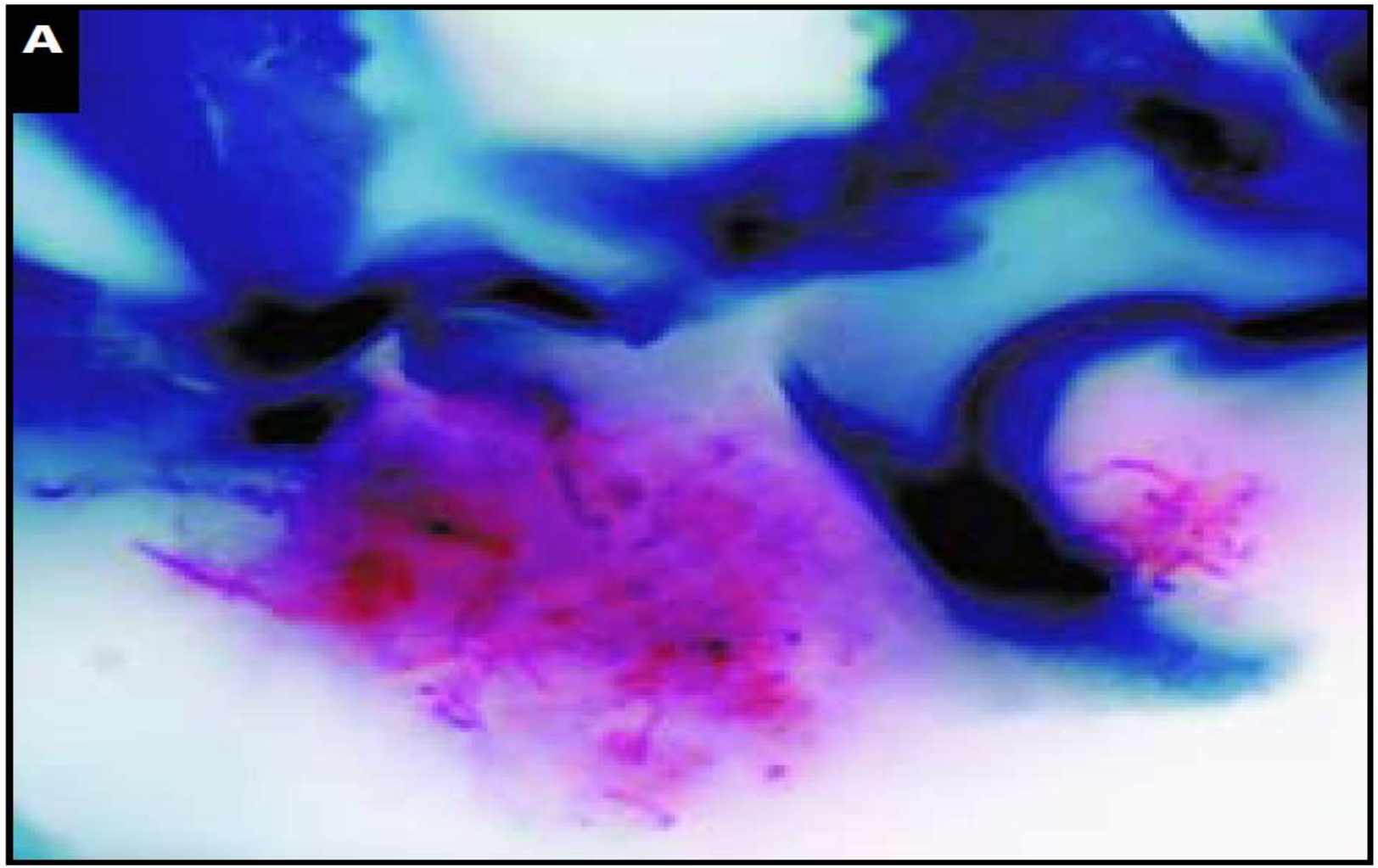
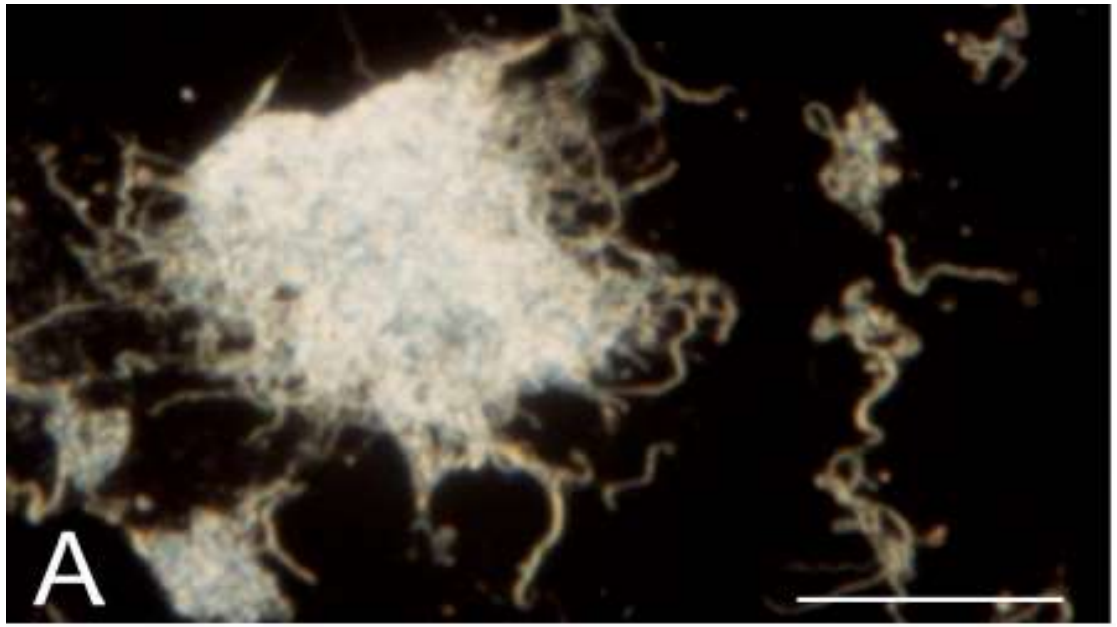
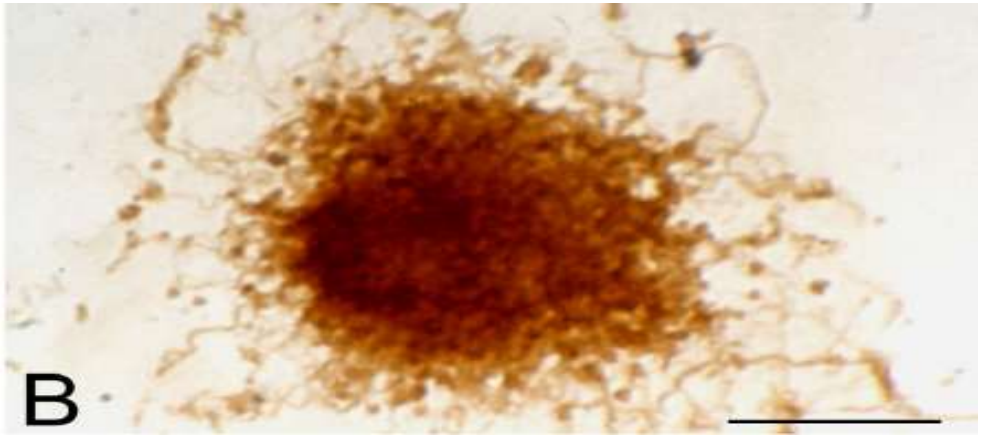
Eisendle K et al 2007

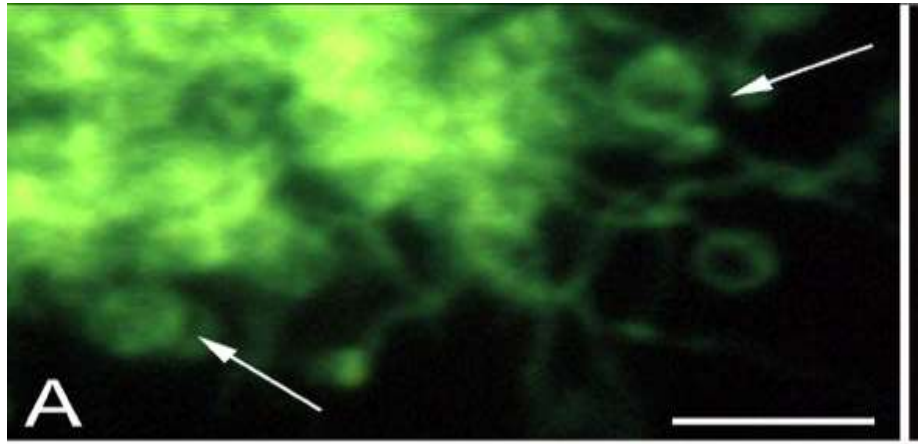


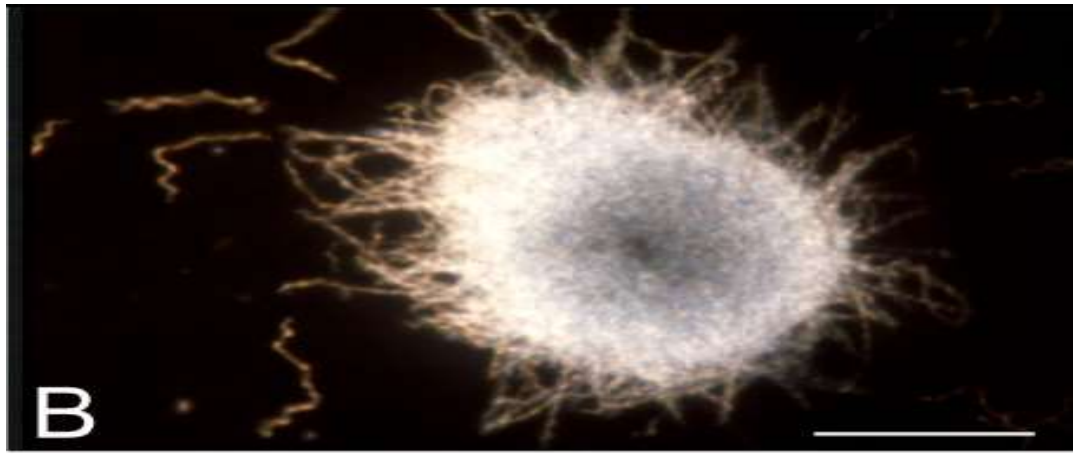
Borrelial lymphocytoma with medusa-like colony of Borrelia

Borrelia burgdorferi "colonies"



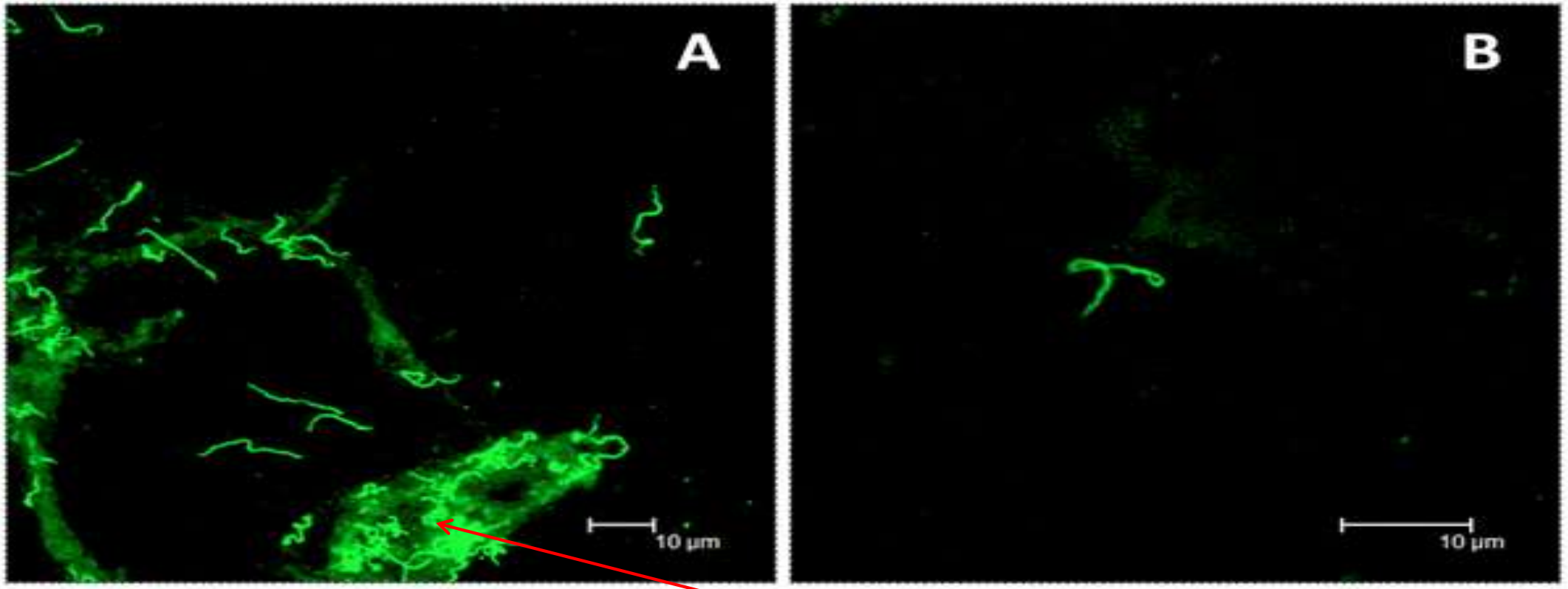






Miklossy J et al 2008

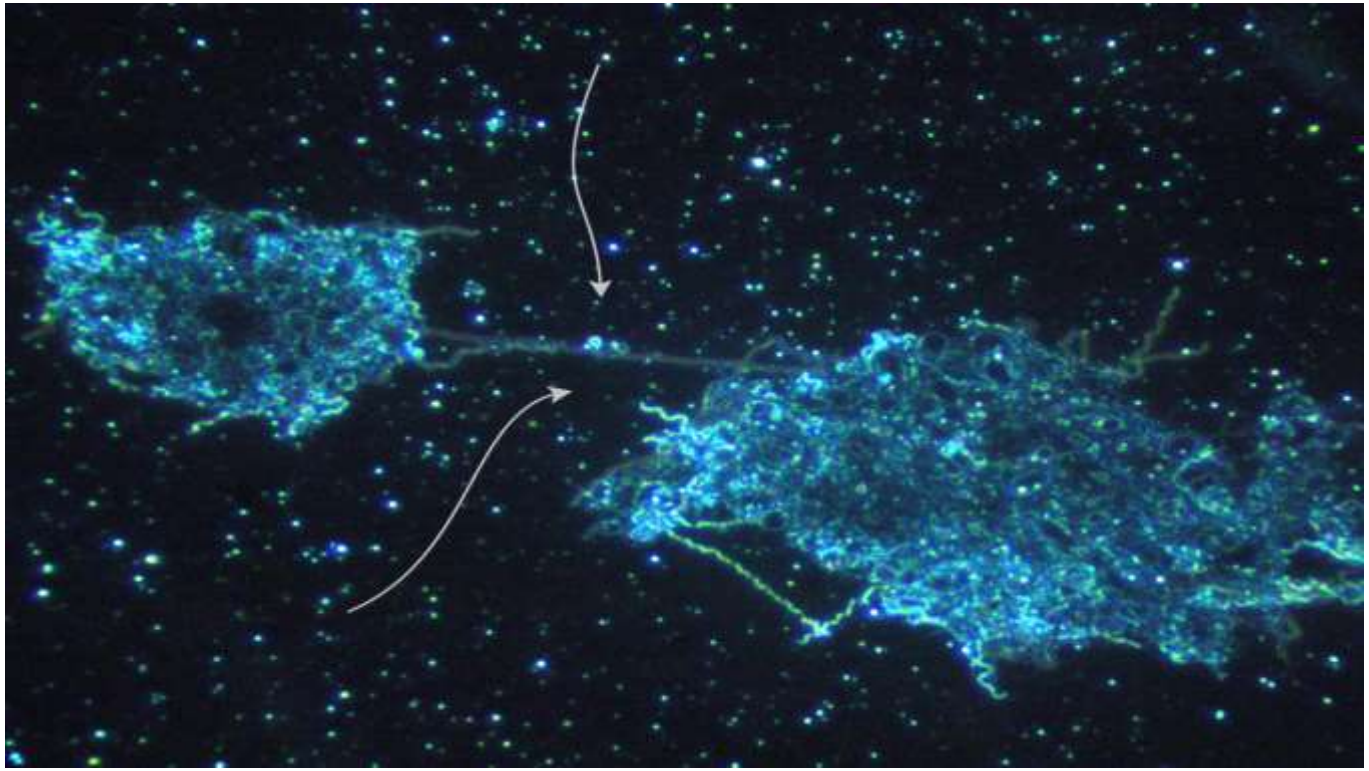
The Rhesus Macaques' study: Embers ME 2012



Borrelia colonies or aggregates?

Fluorescent staining of *Borrelia burgdorferi* spirochetes found in xenodiagnostic tick midgut culture (A)
or tick midgut preparation (B) from treated animals

Borrelia burgdorferi “Photo 51”



Alan MacDonald 2008

MacDonald A, & Sapi E: Biofilms of *Borrelia burgdorferi* in chronic cutaneous borreliosis AJCP 2008 June

- We propose the hypothesis that that *Borrelia burgdorferi* can form biofilm structures in lymphocytomas and acrodermatitis chronica atrophicans.

- Our close examination of these pictures revealed striking similarity to previously published biofilm pictures and our preliminary findings on specific biofilm-like colony formation of *Borrelia burgdorferi* when cultured in the presence of human plasma

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What is Biofilm?

collections of microorganisms (bacteria, yeasts or protozoa) that form on a hard surface (exception floating biofilms)

examples: plaque that forms on teeth and the slime that forms on surfaces in watery areas (shower)

surrounded by slimy secretions: mucoid polysaccharide structure which attaches the community to a surface

estimated that over 90% of bacteria live in biofilm (late Costerton WJ)

Microorganisms found in medical devices

Staphylococcus

Streptococcus

Enterococcus

E. coli

Klebsiella

Pseudomonas

Bacteria may originate from the skin of the patient, or a healthcare worker and tap water

Common biofilms

Dental plaque

Bacterial endocarditis

Urinary tract infections

Cystic fibrosis

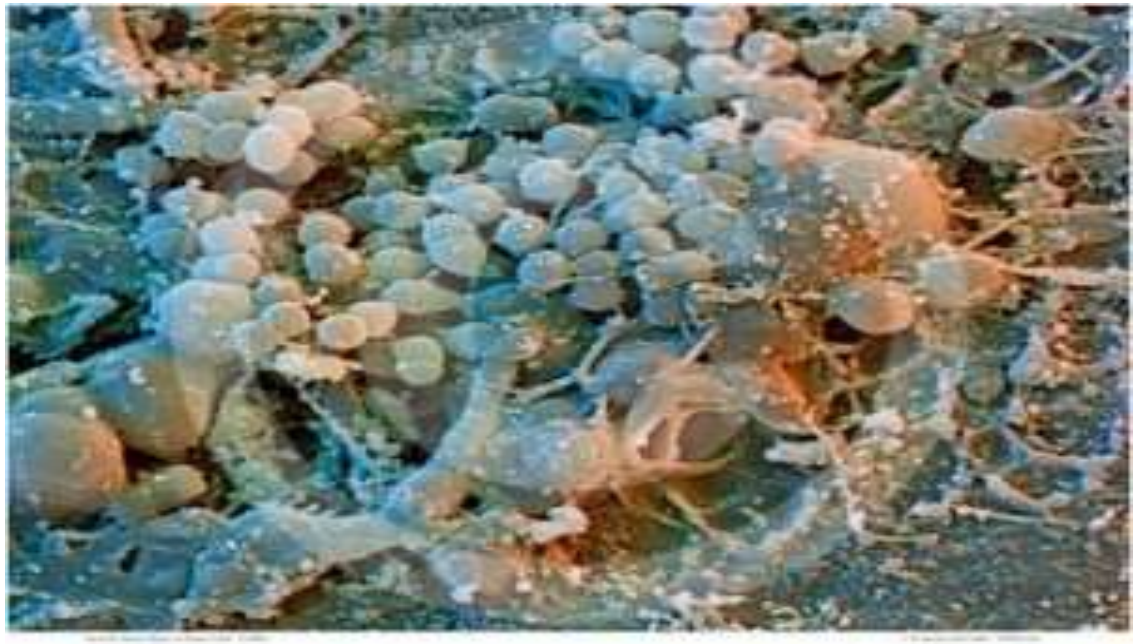
Staphylococcus osteomyelitis

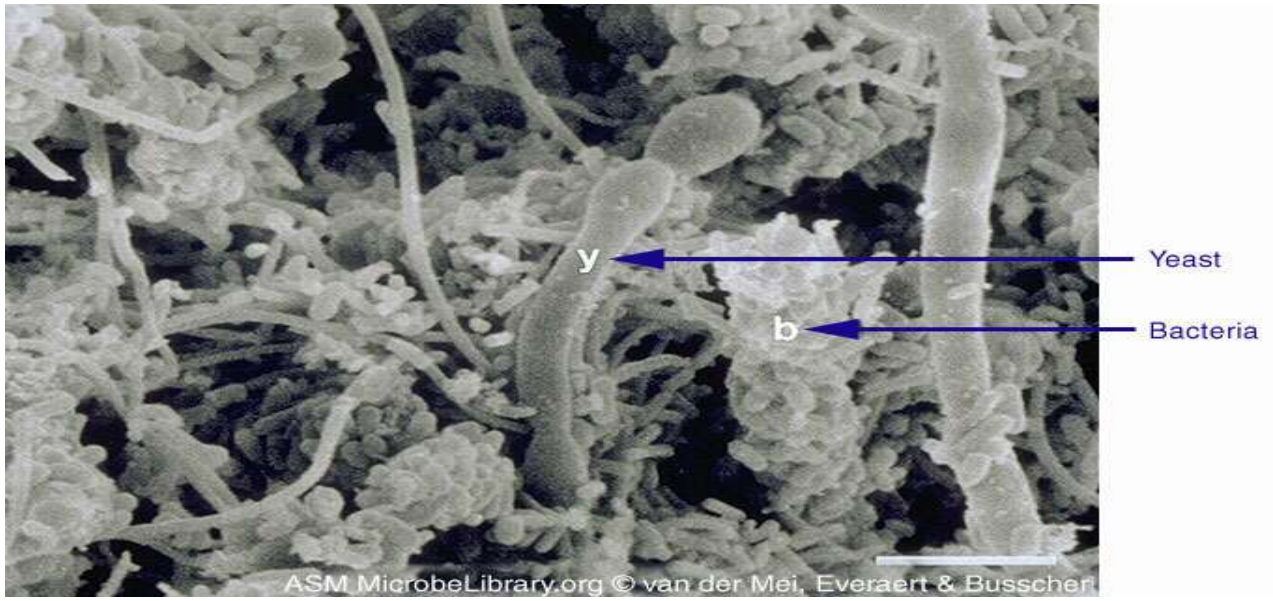
Middle ear infection

Chronic prostatitis

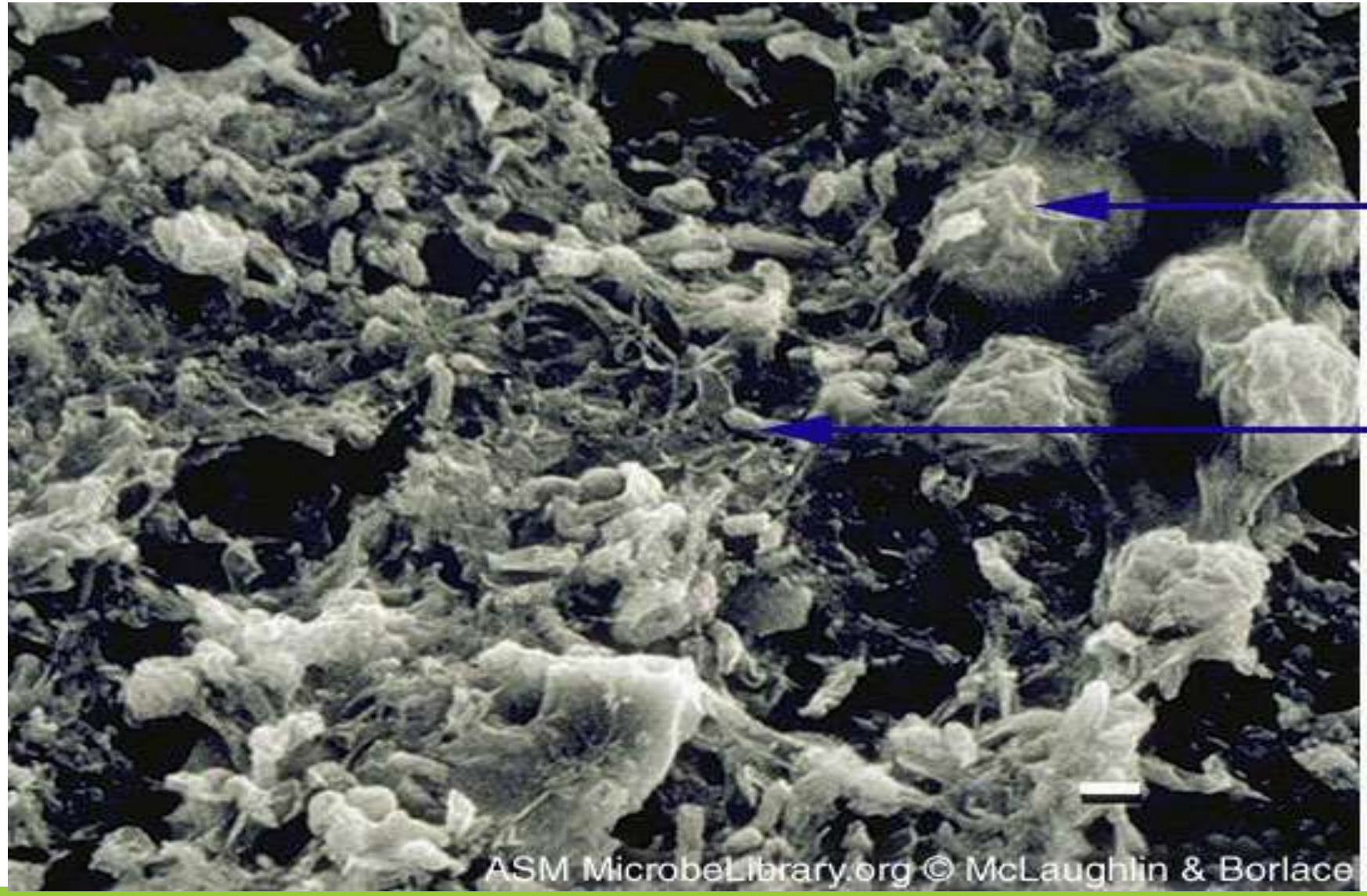
Infectious kidney stones

Dental Plaque: Complex community





Contact lens



Biofilm is like a city

Careful selection of location

Limit settlement of too many organisms

Division of labor (planktonic and sessile cells)

Storage of energy (exopolysaccharides)

Transfer of information (genetic transfer)

Intercellular communication

Emigration when population gets too large for resources

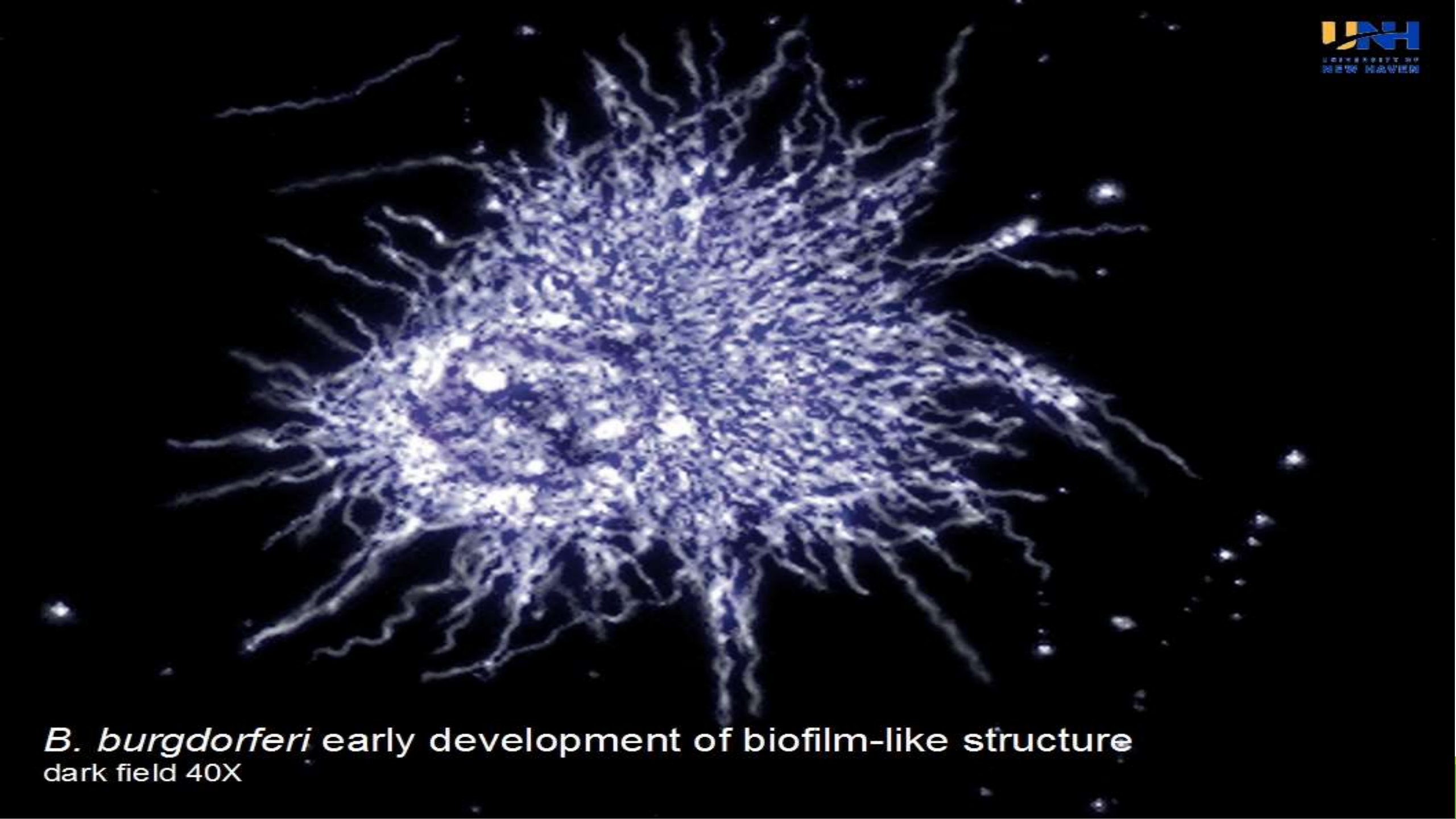
What makes a bacterial colony to a true biofilm?

Loss of motility?

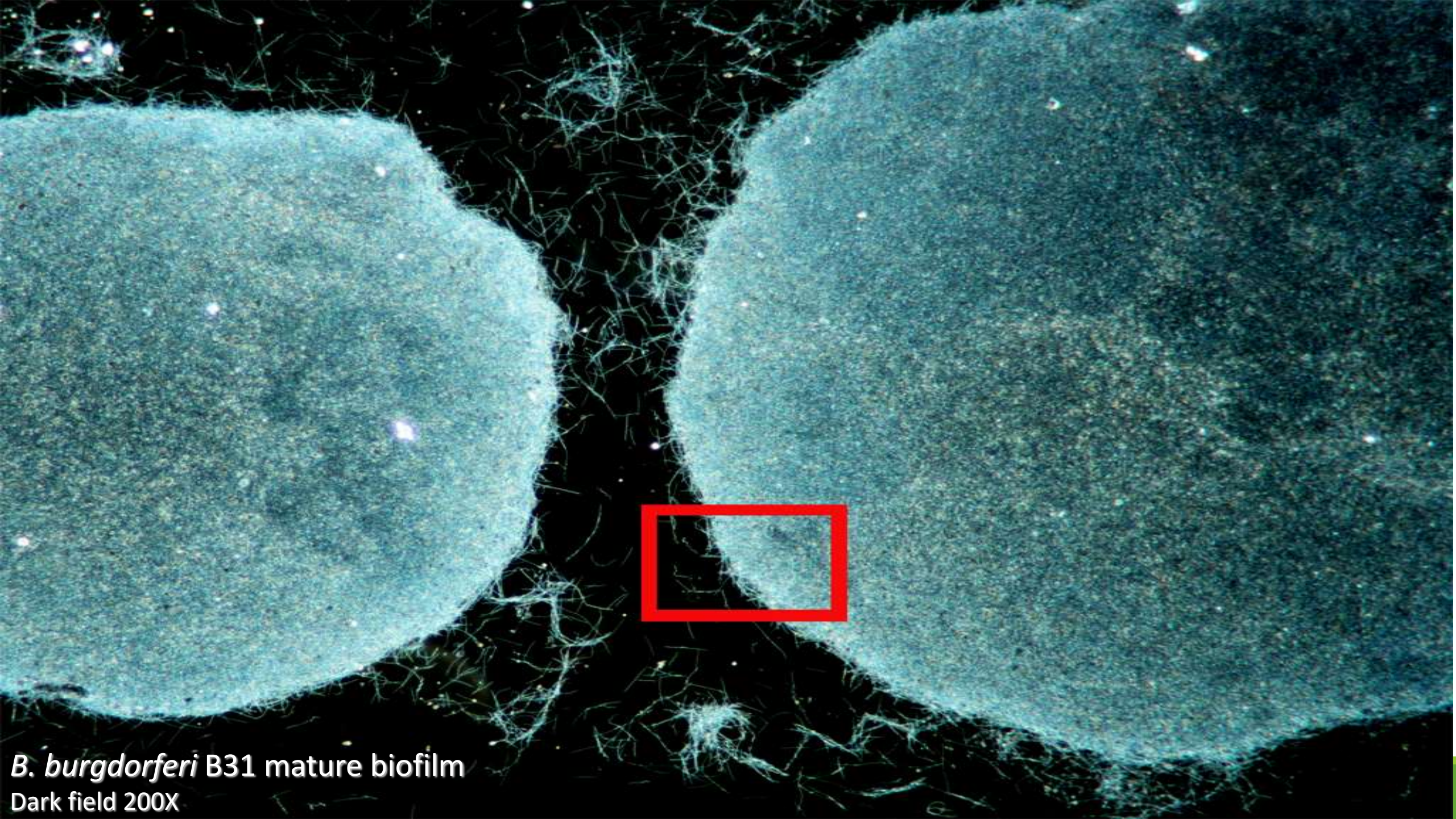
Internal morphological rearrangement

A colony embedded in a matrix of extracellular polymeric substance (EPS) separated by a network of open channels

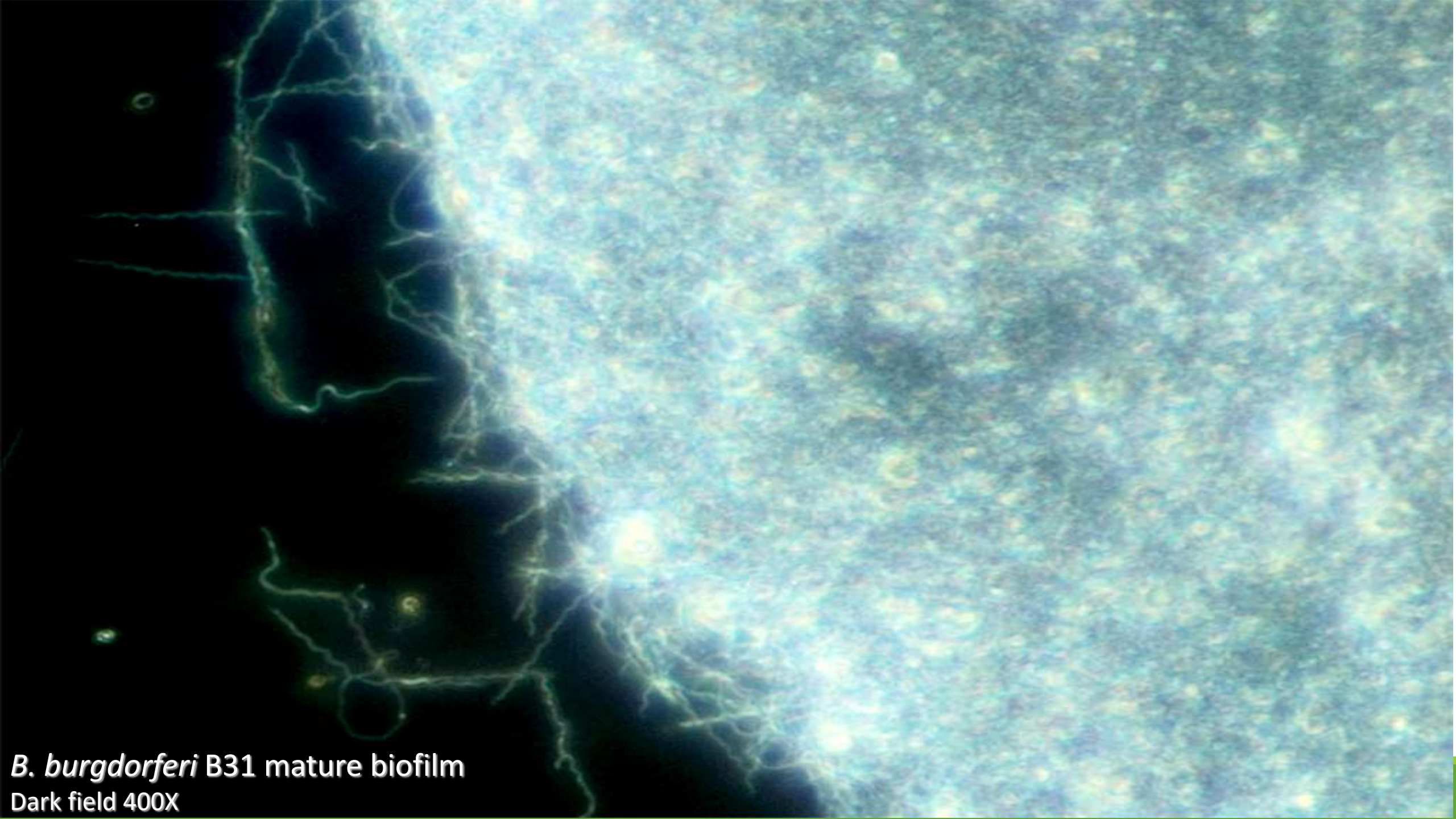
Communication network- quorum sensing



B. burgdorferi early development of biofilm-like structure
dark field 40X

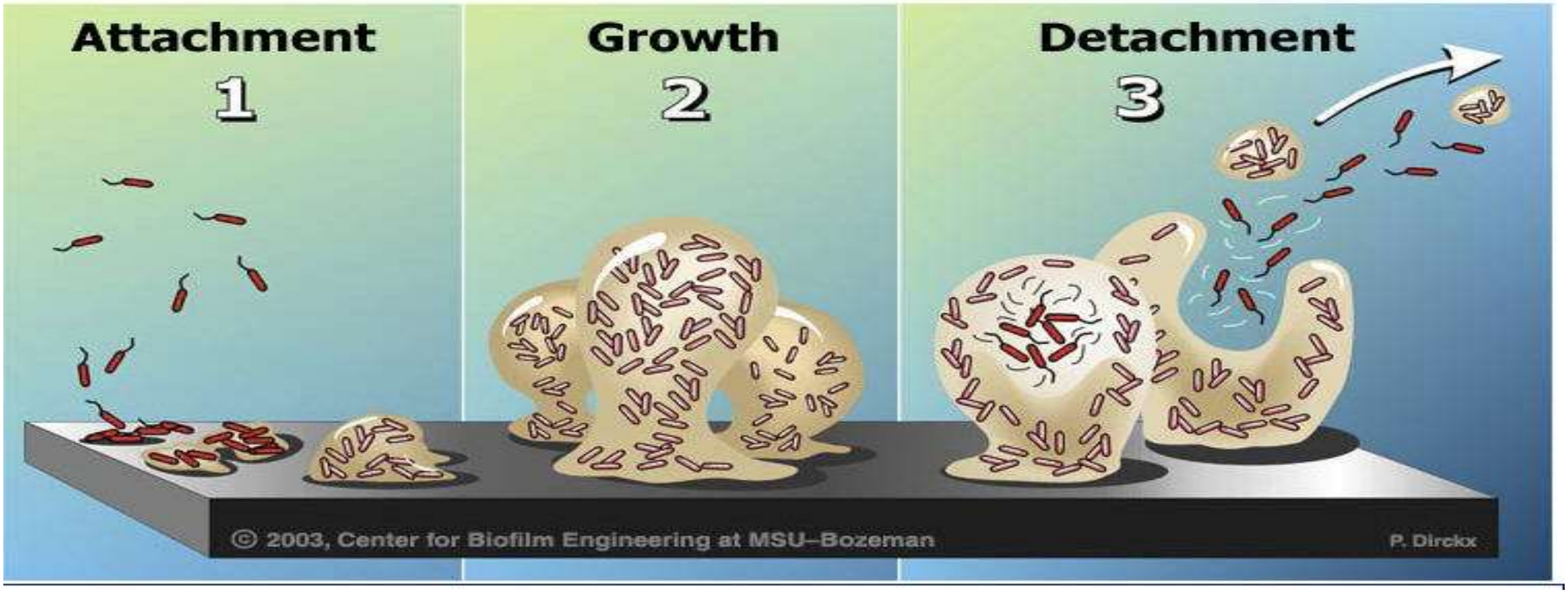


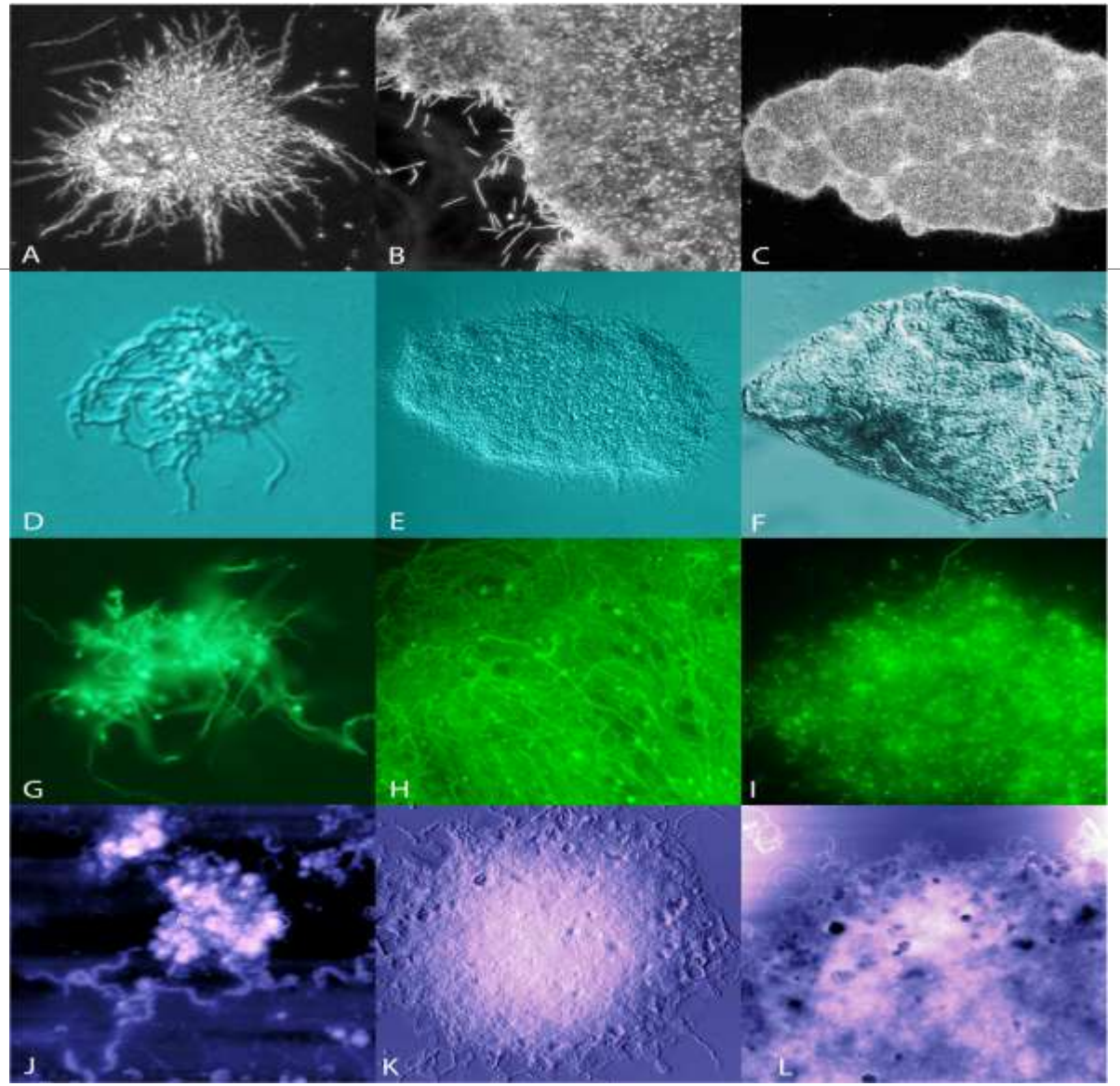
B. burgdorferi B31 mature biofilm
Dark field 200X



B. burgdorferi B31 mature biofilm
Dark field 400X

Stages of biofilm development





Dark-field microscope

DIC

Fluorescent microscope

Atomic Force Microscope

Borrelia burgdorferi biofilm Sapi E at al: PLoS One 2012

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Atomic force microscopy images of live *Borrelia* colony growing on agarose – early development

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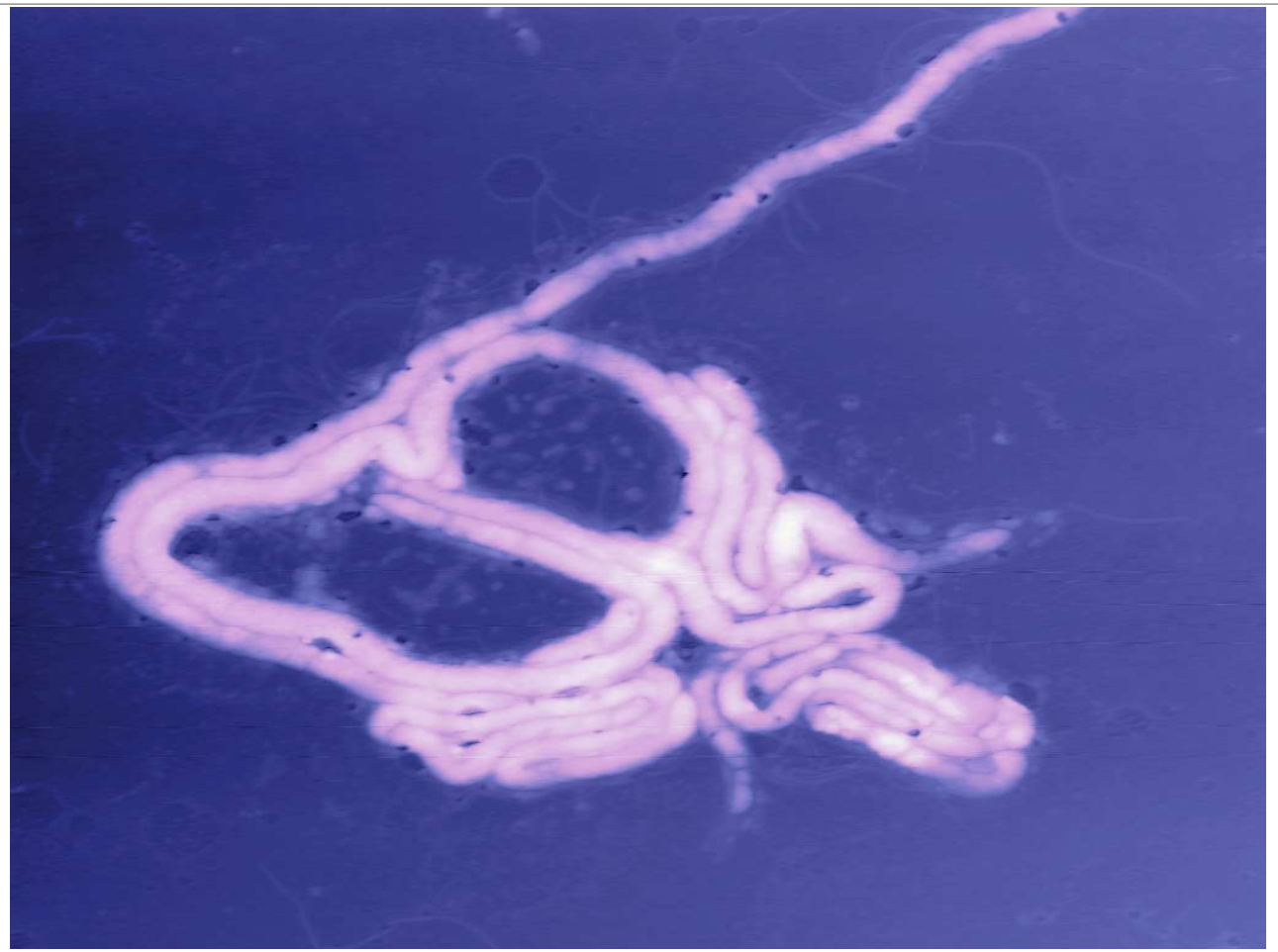
Sapi E at al: PLoS One 2012

Atomic force microscopy images of live *Borrelia* colony growing on agarose – early development



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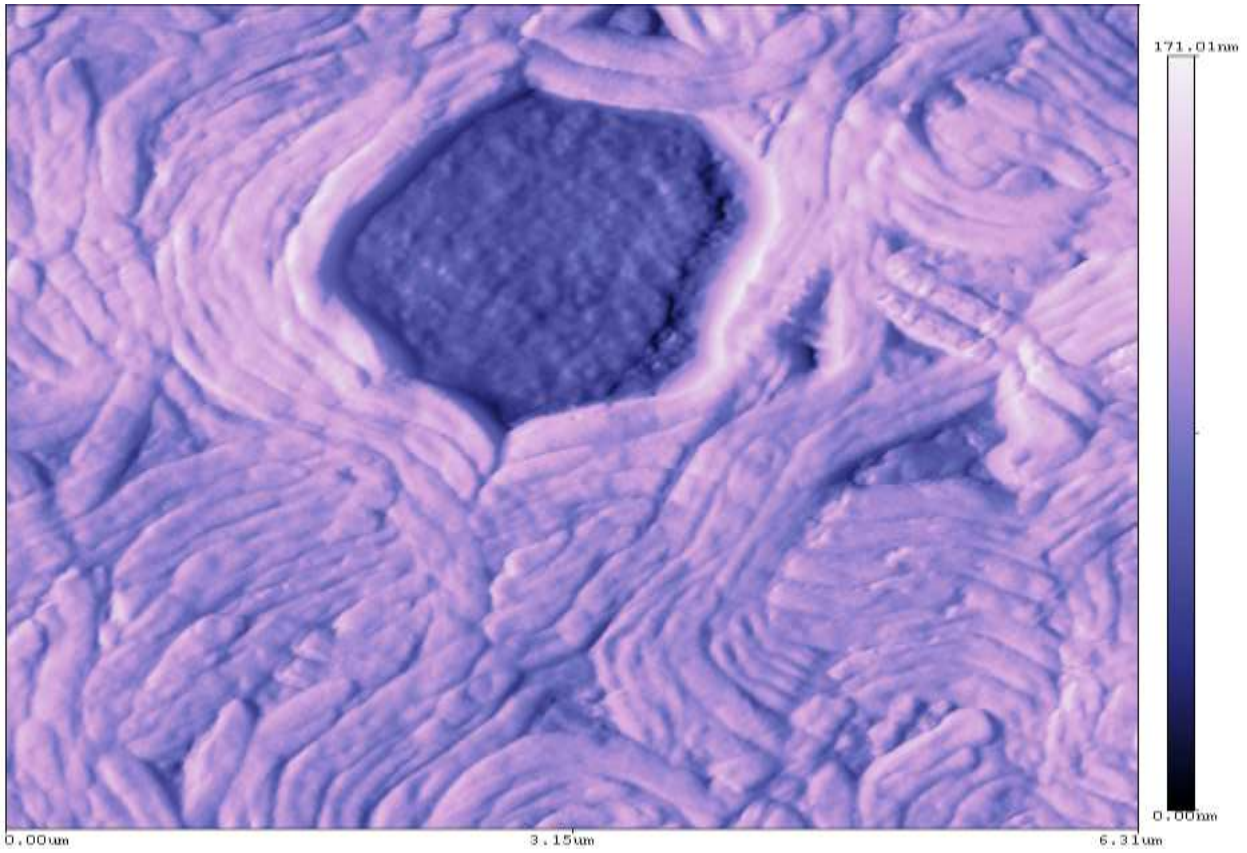
Bastian S, Luecke D, Datar A, Sinha S and Sapi E 2011

Atomic force microscopy images of live *Borrelia* colony growing on agarose – mid-phase development



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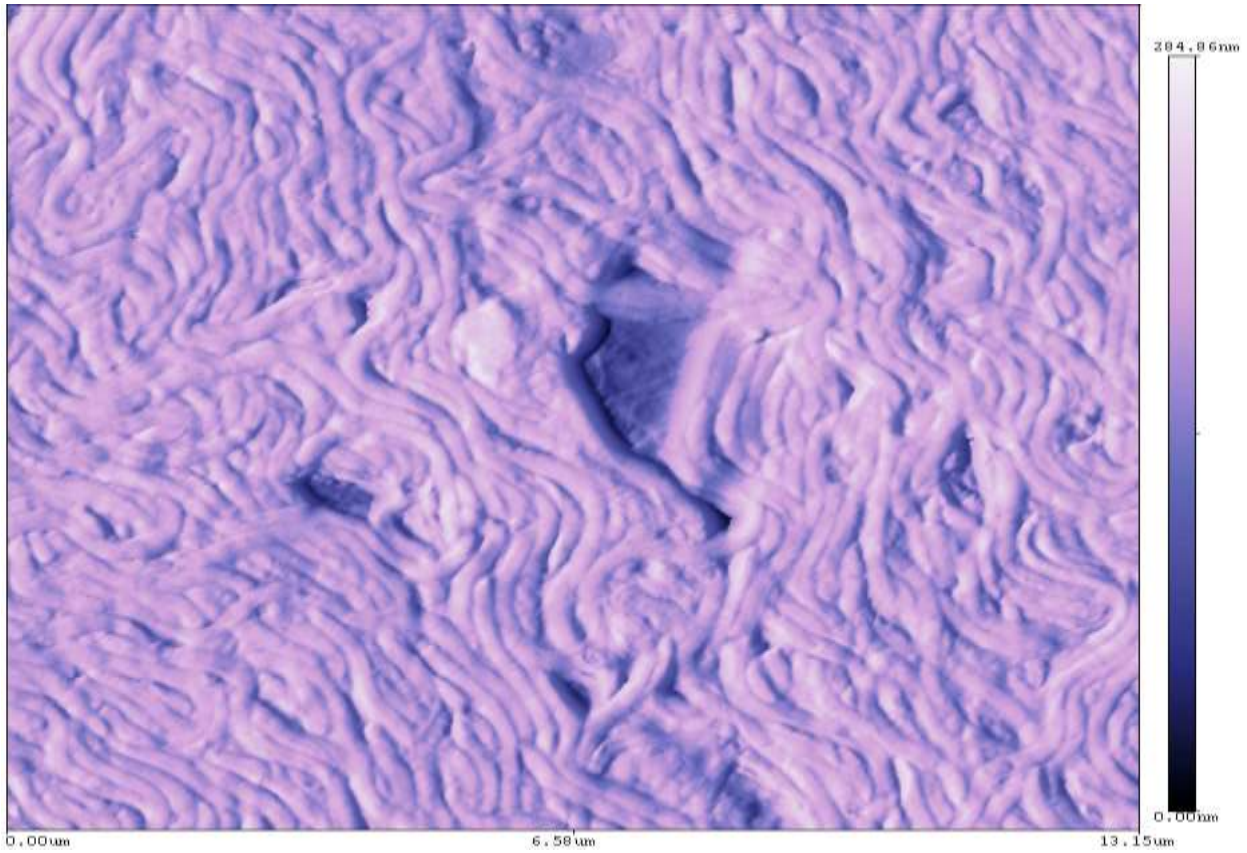
Bastian S, Luecke D, Datar A, Sinha S and Sapi E 2011

Atomic force microscopy images of live *Borrelia* colony growing on agarose – midphase development – 2 days later



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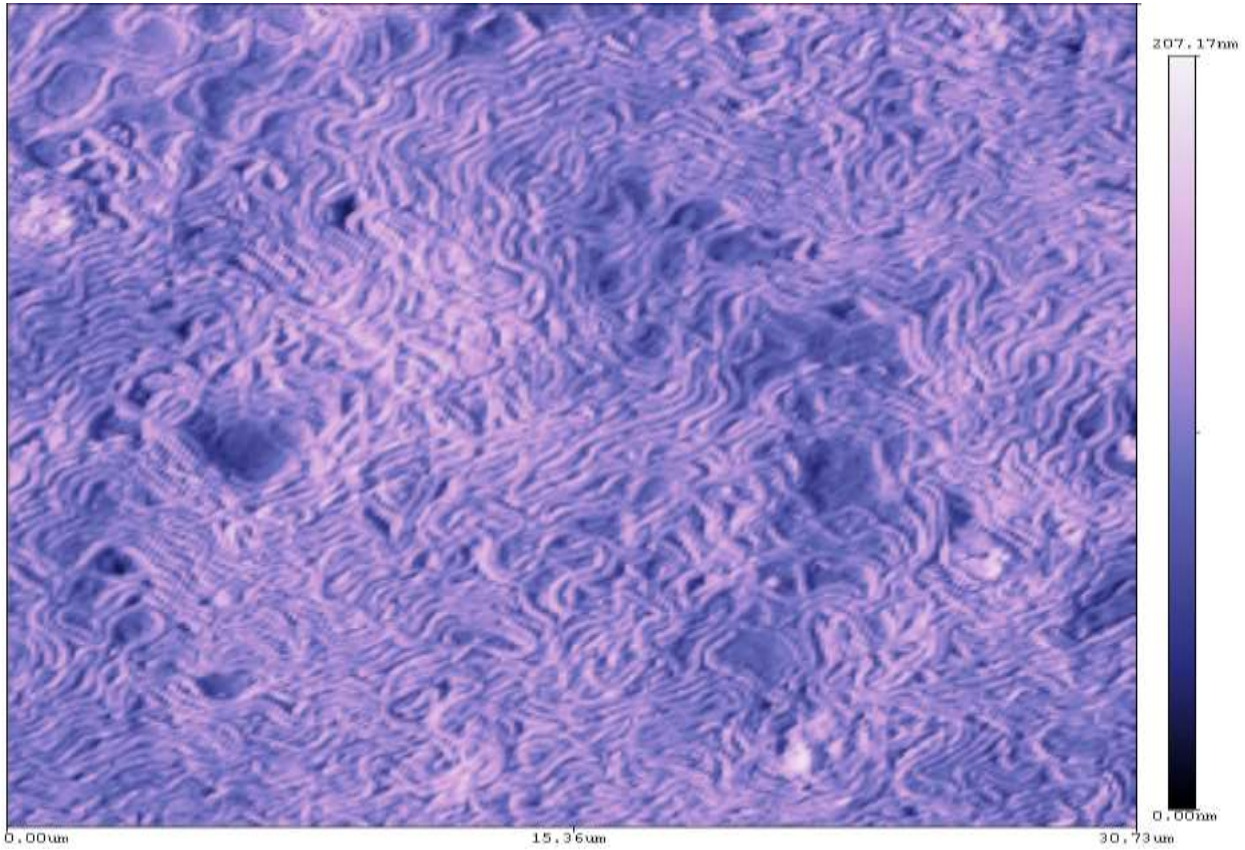
Bastian S, Luecke D, Datar A, Sinha S and Sapi E 2011

Atomic force microscopy images of live *Borrelia* colony growing on agarose –late phase



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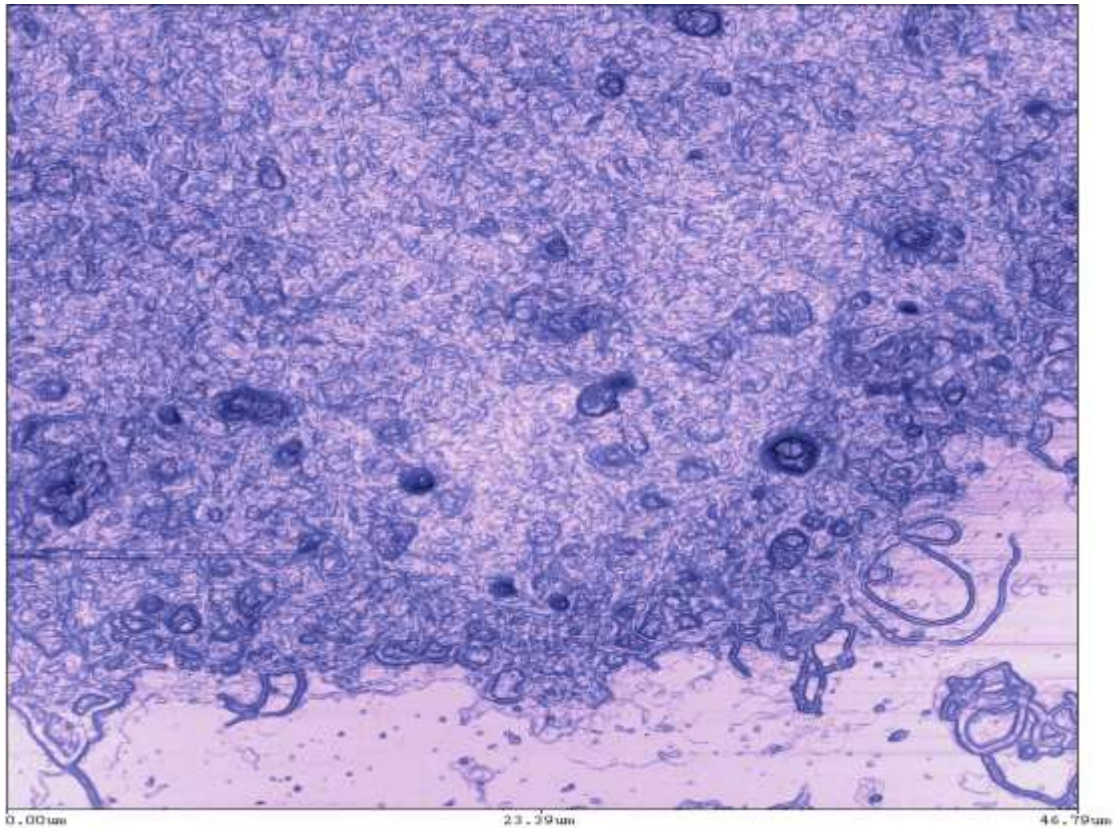
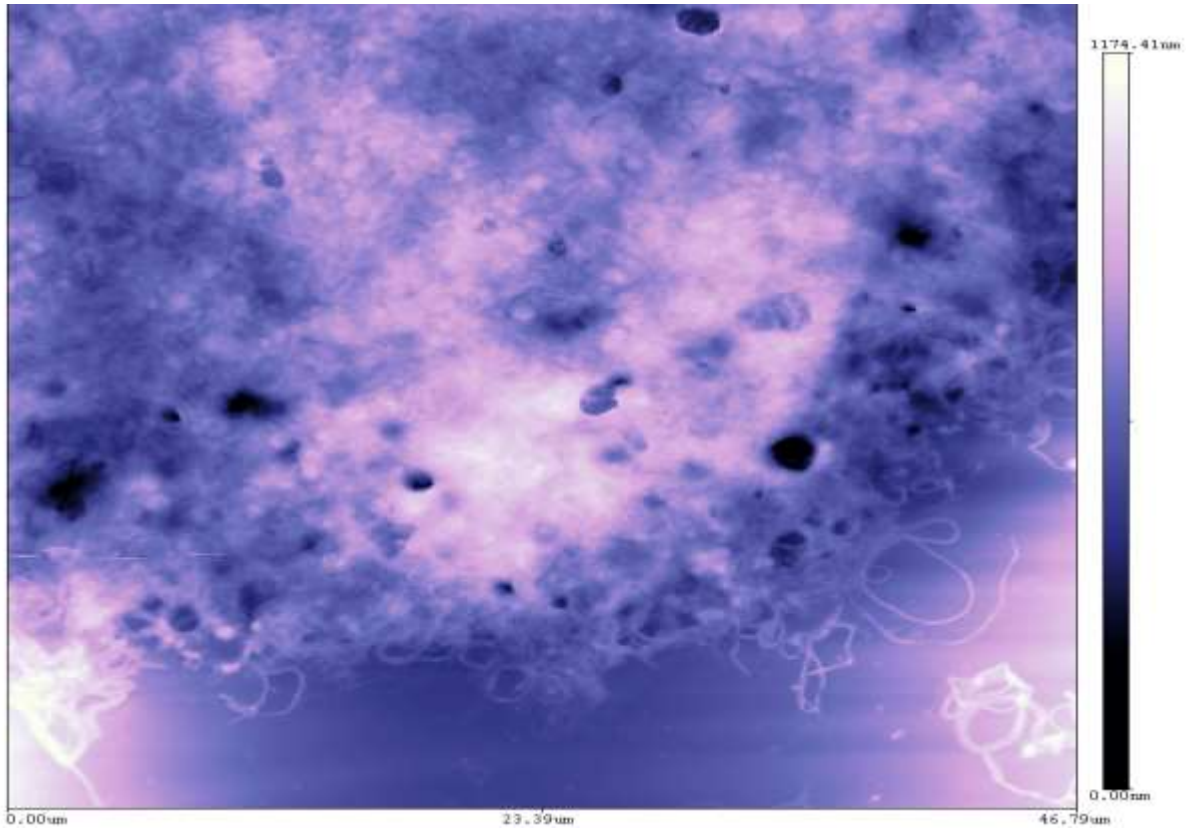
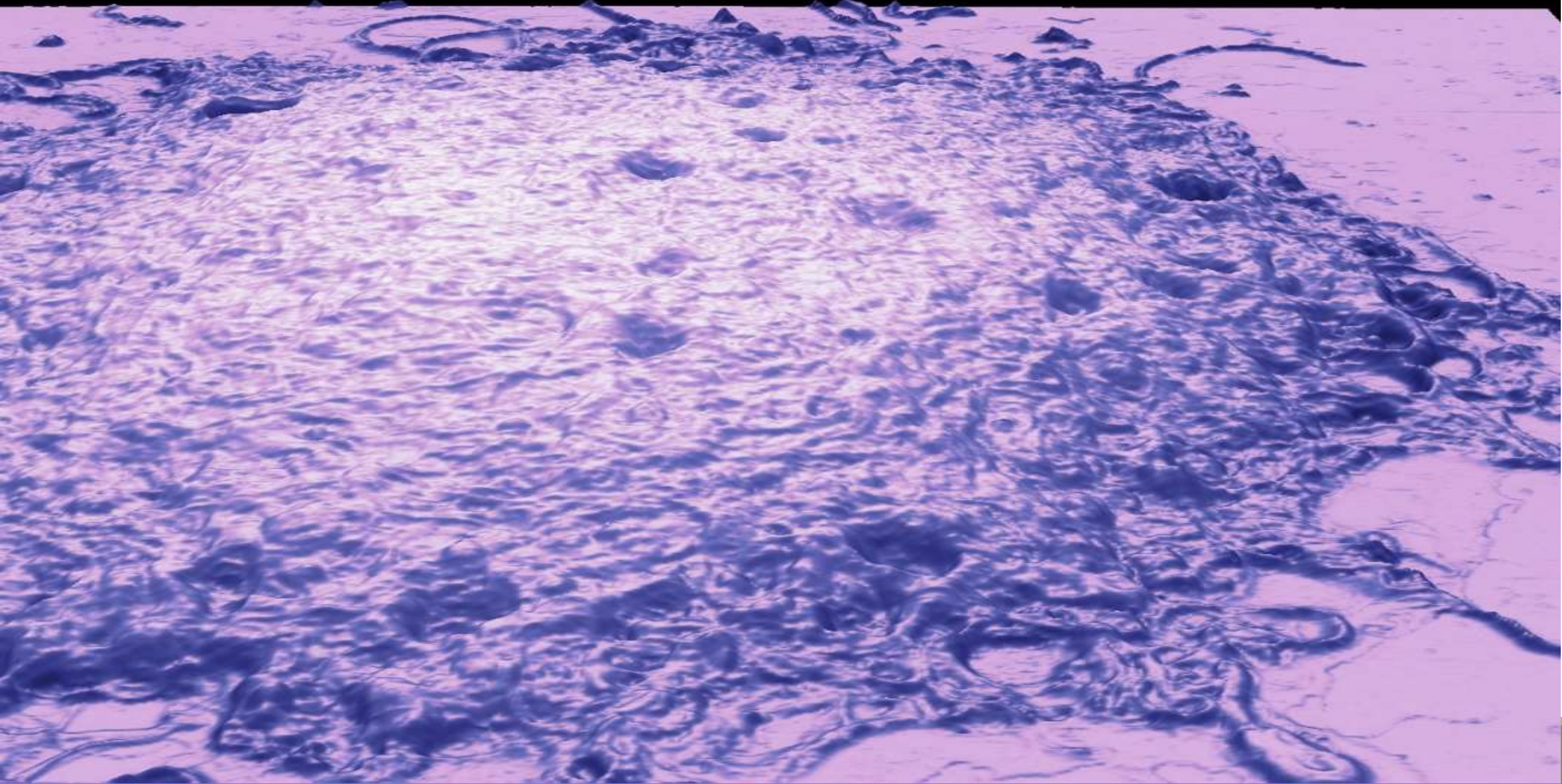


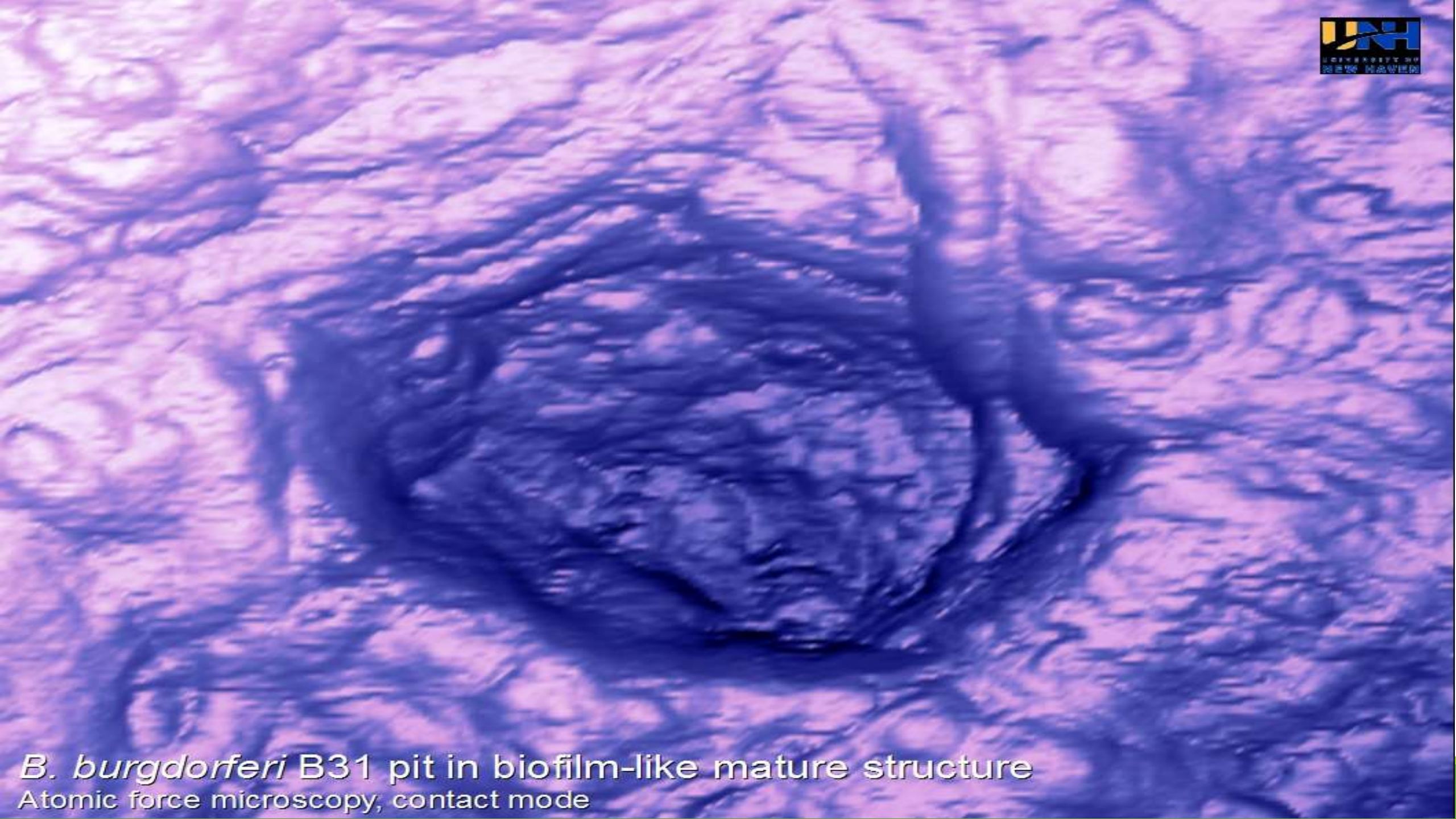


Luecke D, Bastian S, Datar A, Sinha S and Sapi E 2011

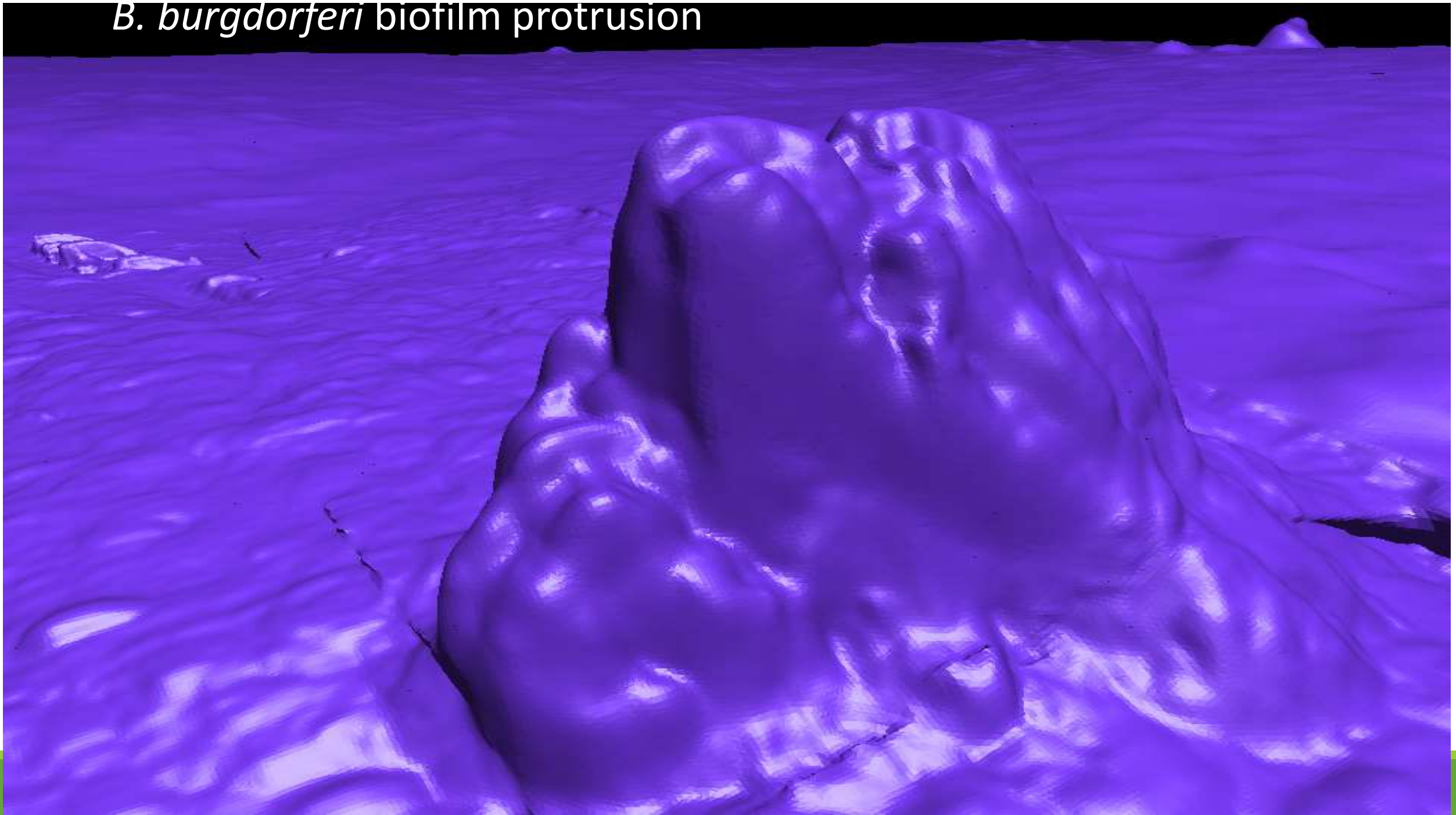
B. burgdorferi B31 biofilm-like mature structure

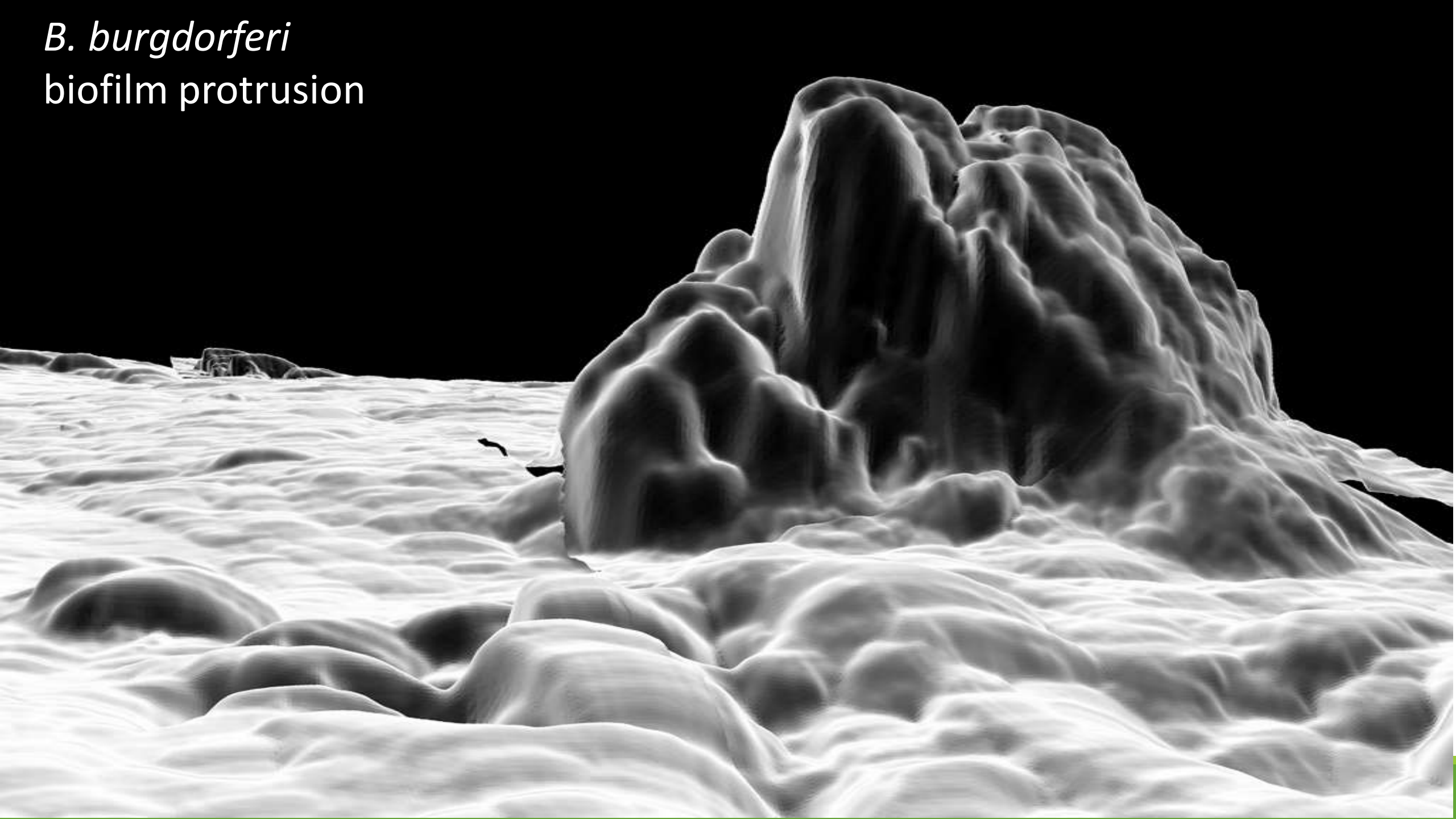
Atomic force microscopy, contact mode

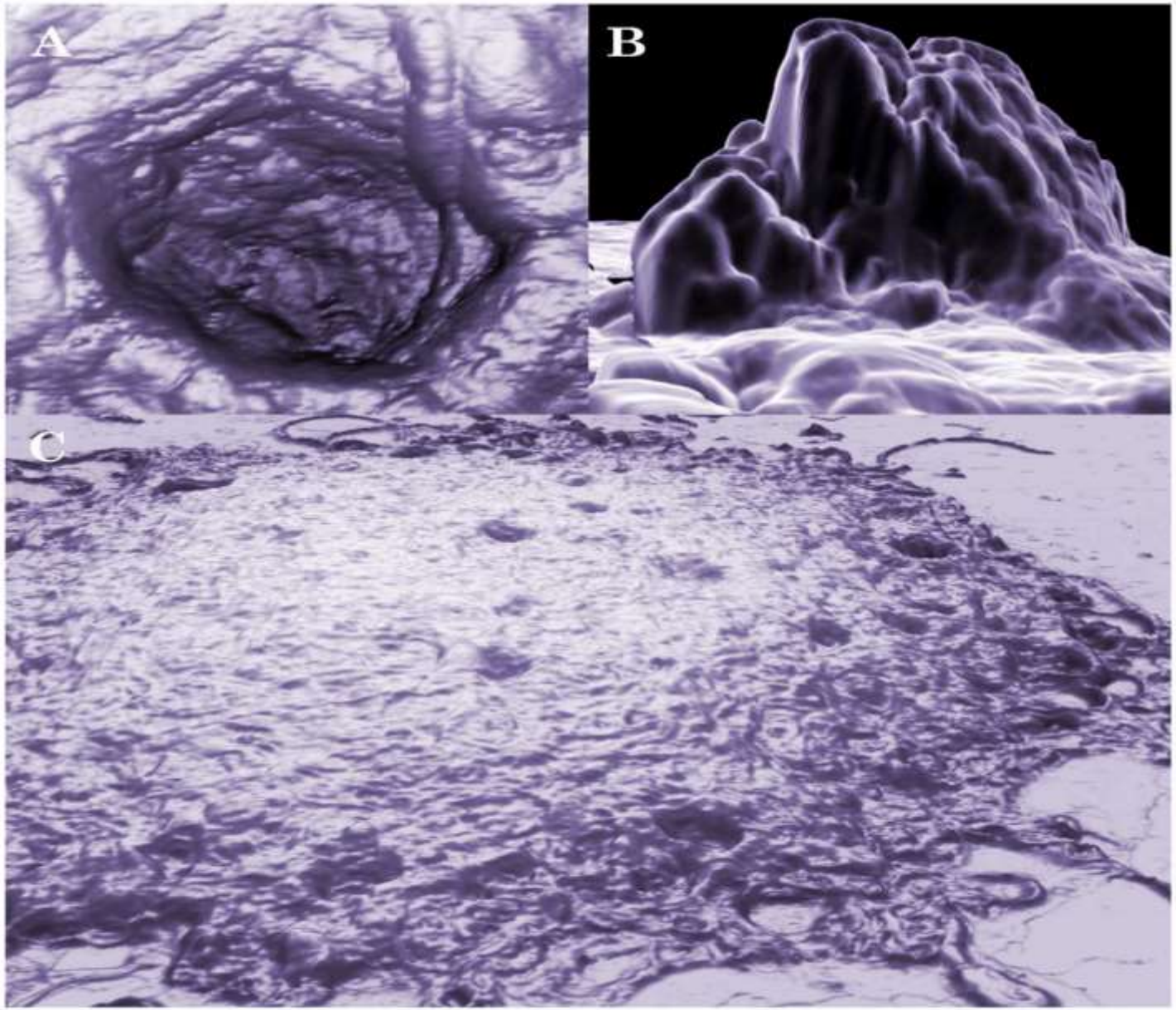



B. burgdorferi B31 pit in biofilm-like mature structure
Atomic force microscopy, contact mode

B. burgdorferi biofilm protrusion




B. burgdorferi
biofilm protrusion



Sapi E et al 2012 PLoS ONE

Extracellular Polymeric Substances

The EPS matrix: The "house of biofilm cells"

Composed of mucopolysaccharides (slime), proteins (enzymes) glycoproteins, glycolipids, extracelullar DNA

Some of these polysaccharides are polyanionic (like uronic acid) – can bind to calcium and magnesium

Costerton JW and Irvin RT 1984, Flemming HC 2007

Spicer Meyer aldehyde fuchsine – alcian blue stain sequence for mucopolysaccharides

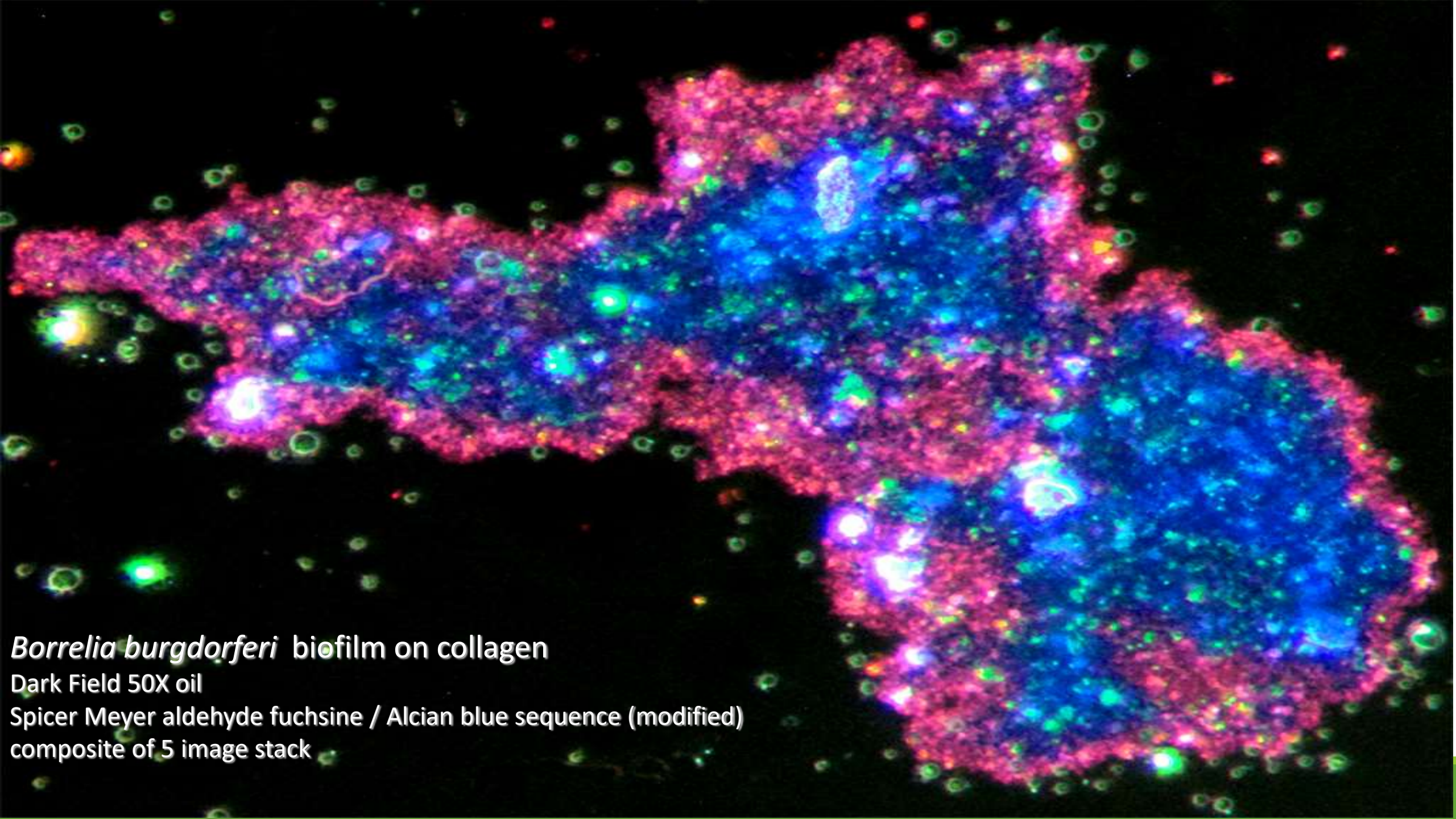
Aldehyde fuchsine:

Stains acidic sulfated mucins

Alcian blue:

Stains remaining non-sulfated mucins, carboxylated mucopolysaccharides

Spicer & Meyer, 1960


Borrelia burgdorferi biofilm on collagen
Dark Field 50X oil
Spicer Meyer aldehyde fuchsine / Alcian blue sequence (modified)
composite of 5 image stack

Borrelia EPS component: alginate?

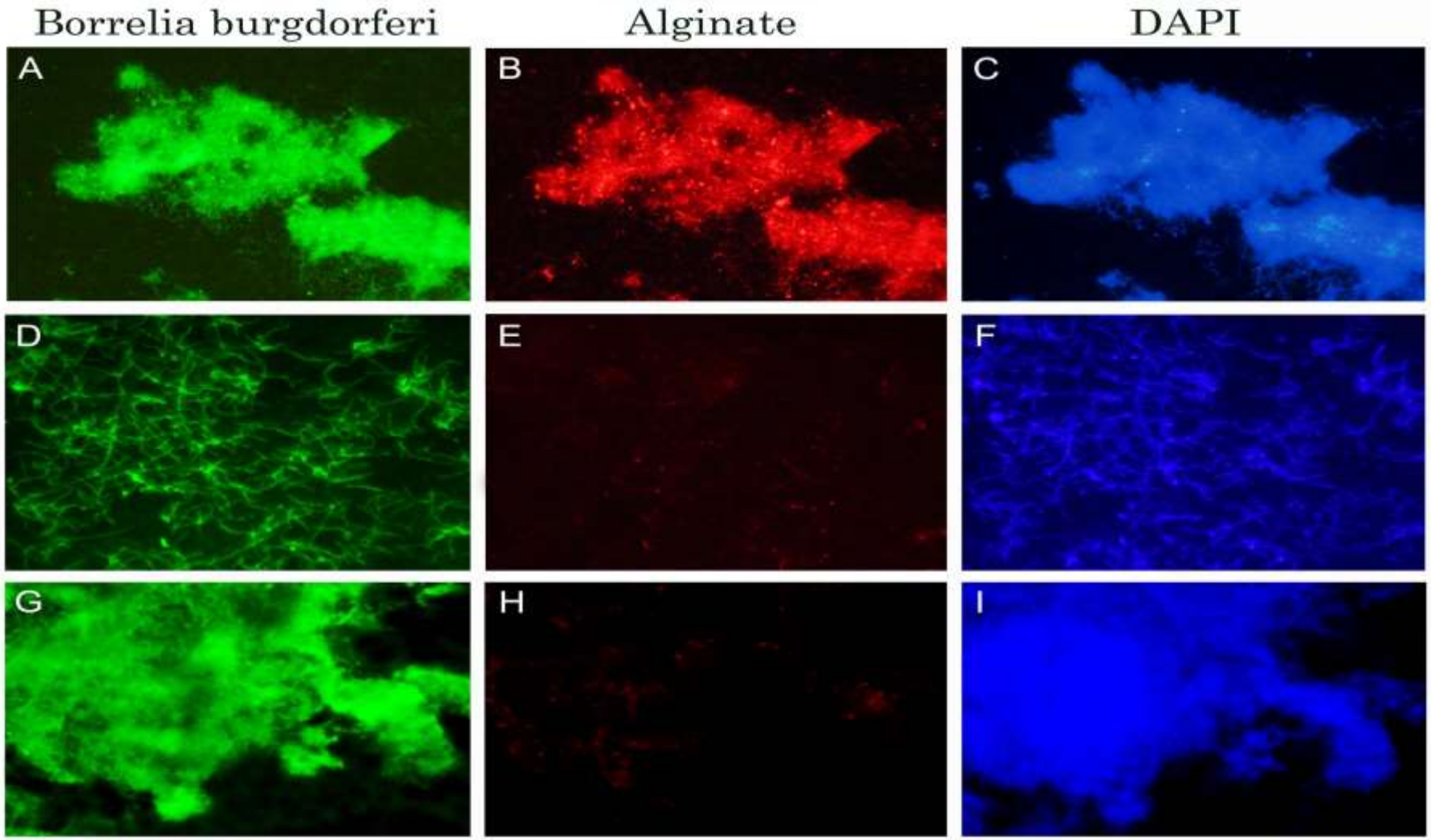
Alginate is well established as a viable primary EPS compound

Composed primarily of **polyuronic acid polymer – alginate** (Kjelleberg S 2007)

Chemical analysis of two varieties of slime produced by *Pseudomonas aeruginosa* (*Murakama K 1973*)

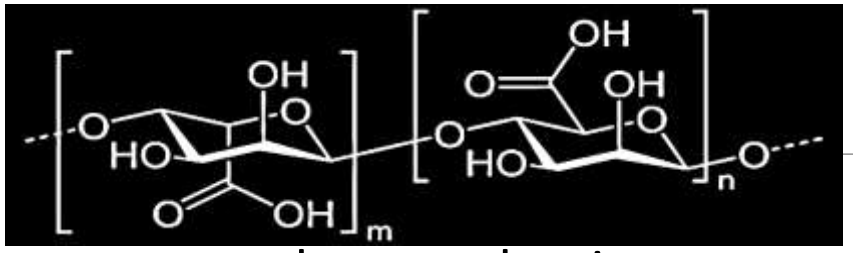
"The exopolysaccharide alginate protects *Pseudomonas aeruginosa* biofilm bacteria from macrophage killing." *(Leid JG 2005)*

Alginate on the surfaces of *Borrelia* aggregates



Sapi E et al 2012 PLoS ONE

EPS: alginate and calcium



copolymer subunits

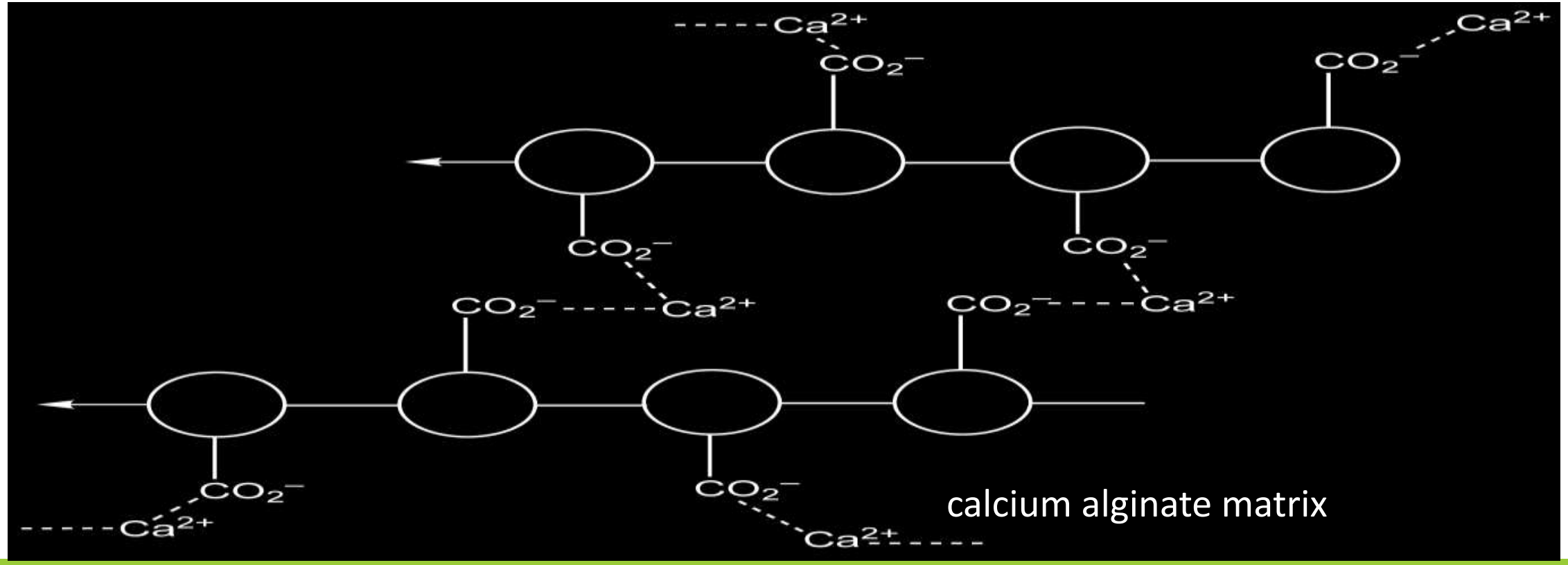
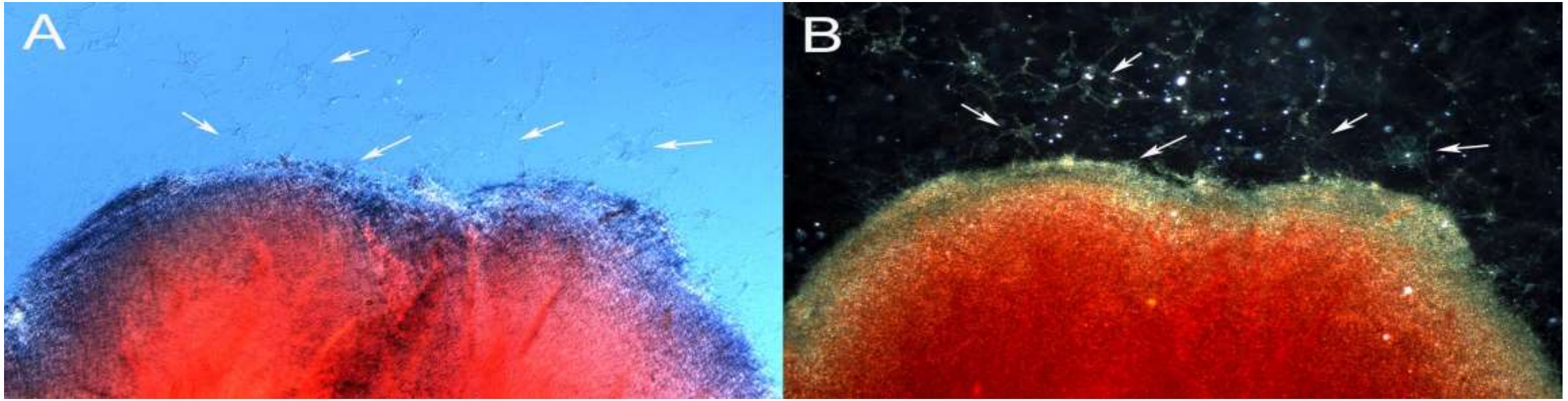


image: RSC.org

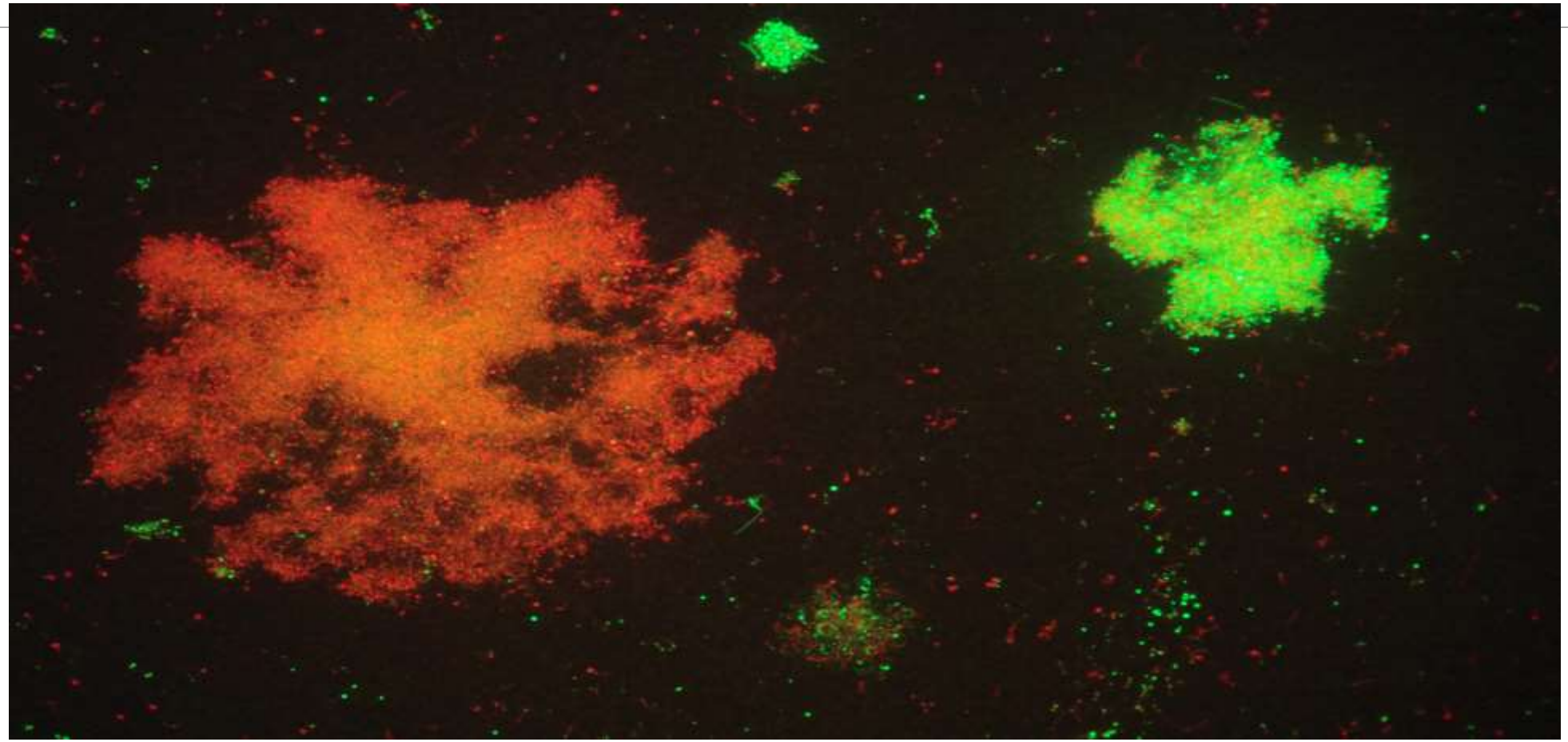
Calcium on the surface of *Borrelia* aggregates



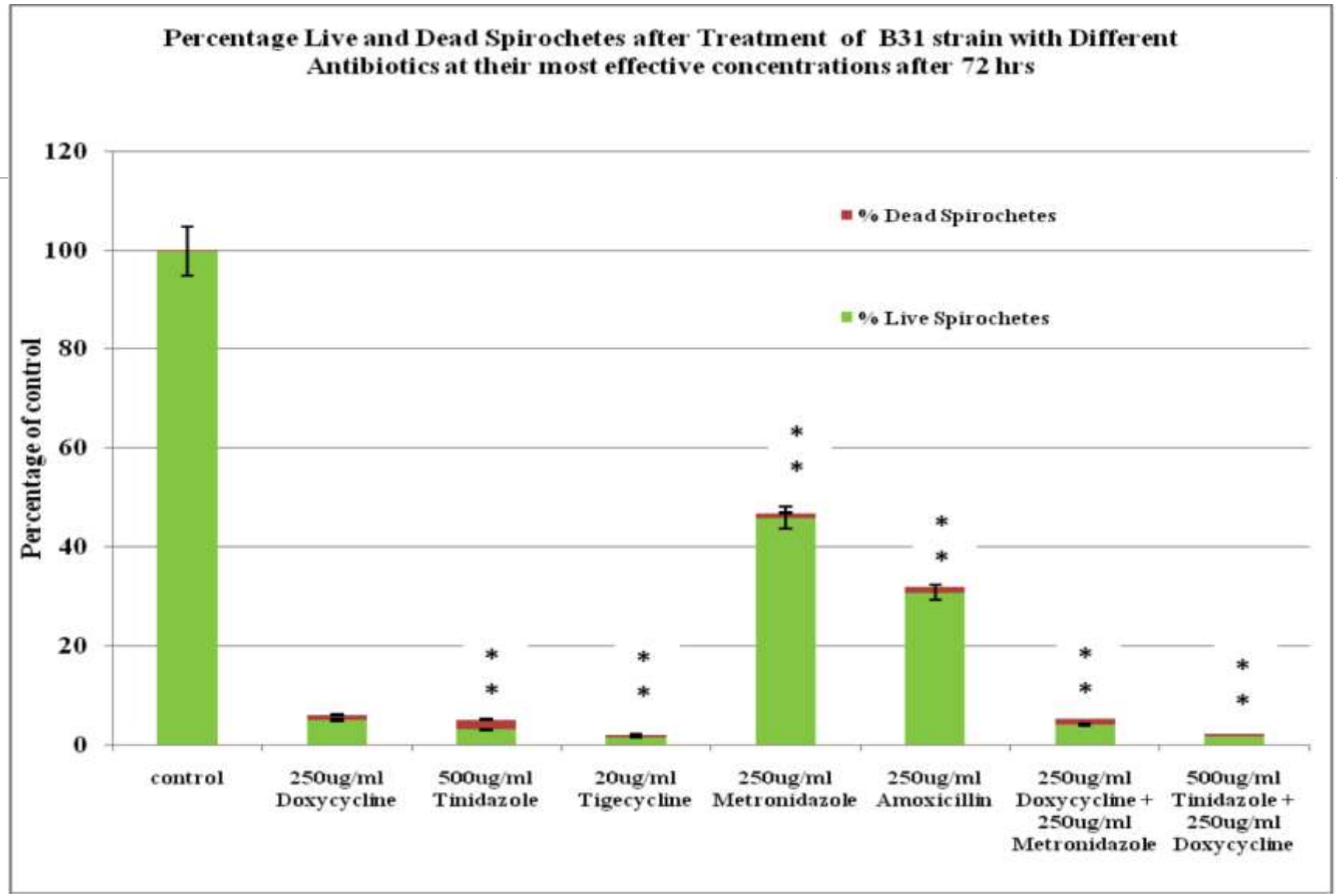
Borrelia burgdorferi B31 strain large aggregate surrounded by individual spirochetes and several small aggregates stained with the calcium-detecting stain Alizarin. Red coloration indicates presence of calcium, by differential interference contrast (Panel A) and dark field microscopy (Panel B).

Sapi E et al PLoS ONE 2012

Borrelia burgdorferi treated with 25 microgram/ml of doxycyline for 3 weeks



Luecke DF, Kaur N, Datar A and E Sapi unpublished data 2012



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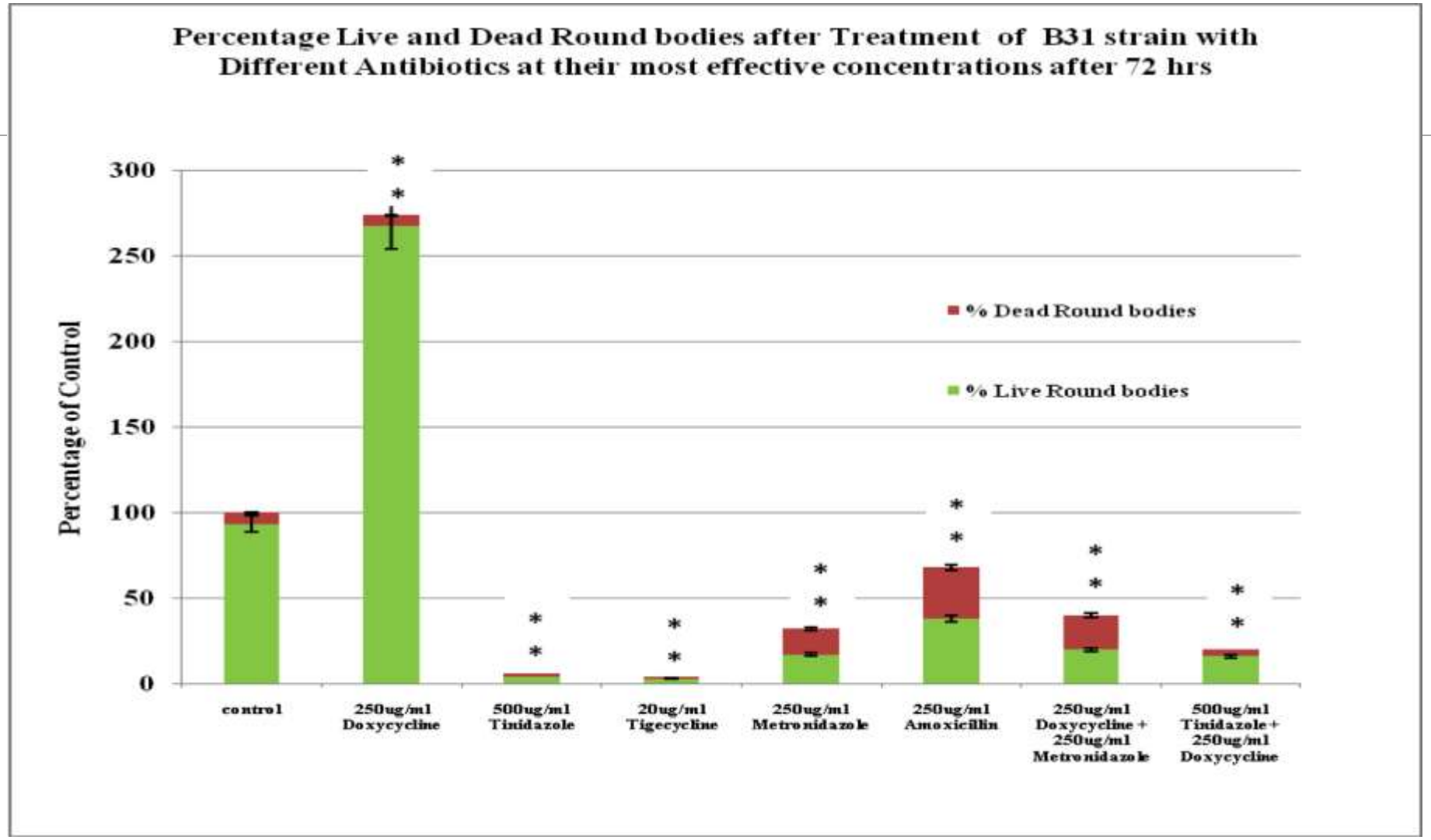
Kaur N, Sapi E 2012 unpublished data



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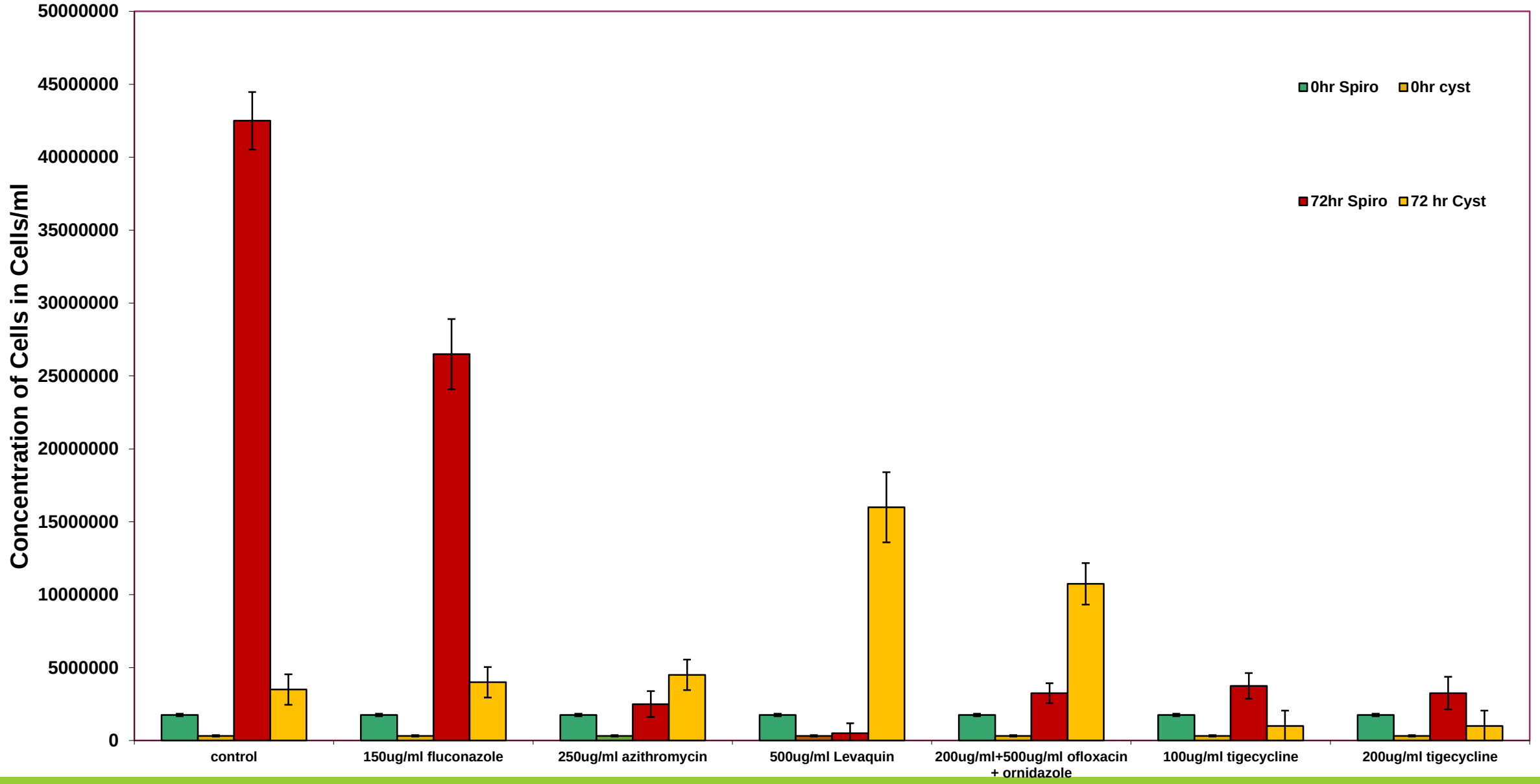
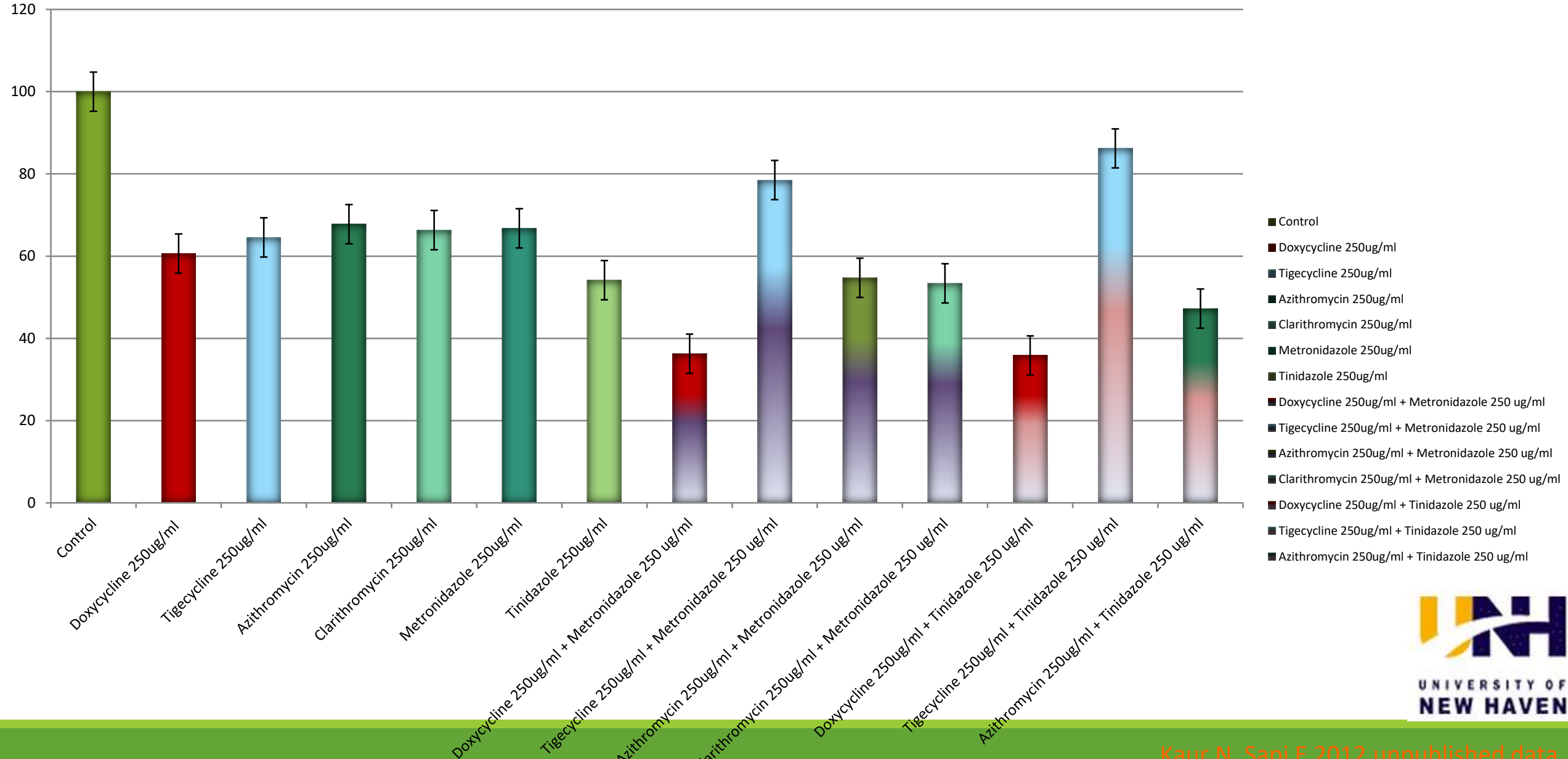
Kaur N, Sapi E 2012 unpublished data

Sensitivity of Borrelia forms to different antibiotics



Kaur N, Sapi E 2012 unpublished data

Effect of different antibiotics after treatment of Bb biofilm for 3 days



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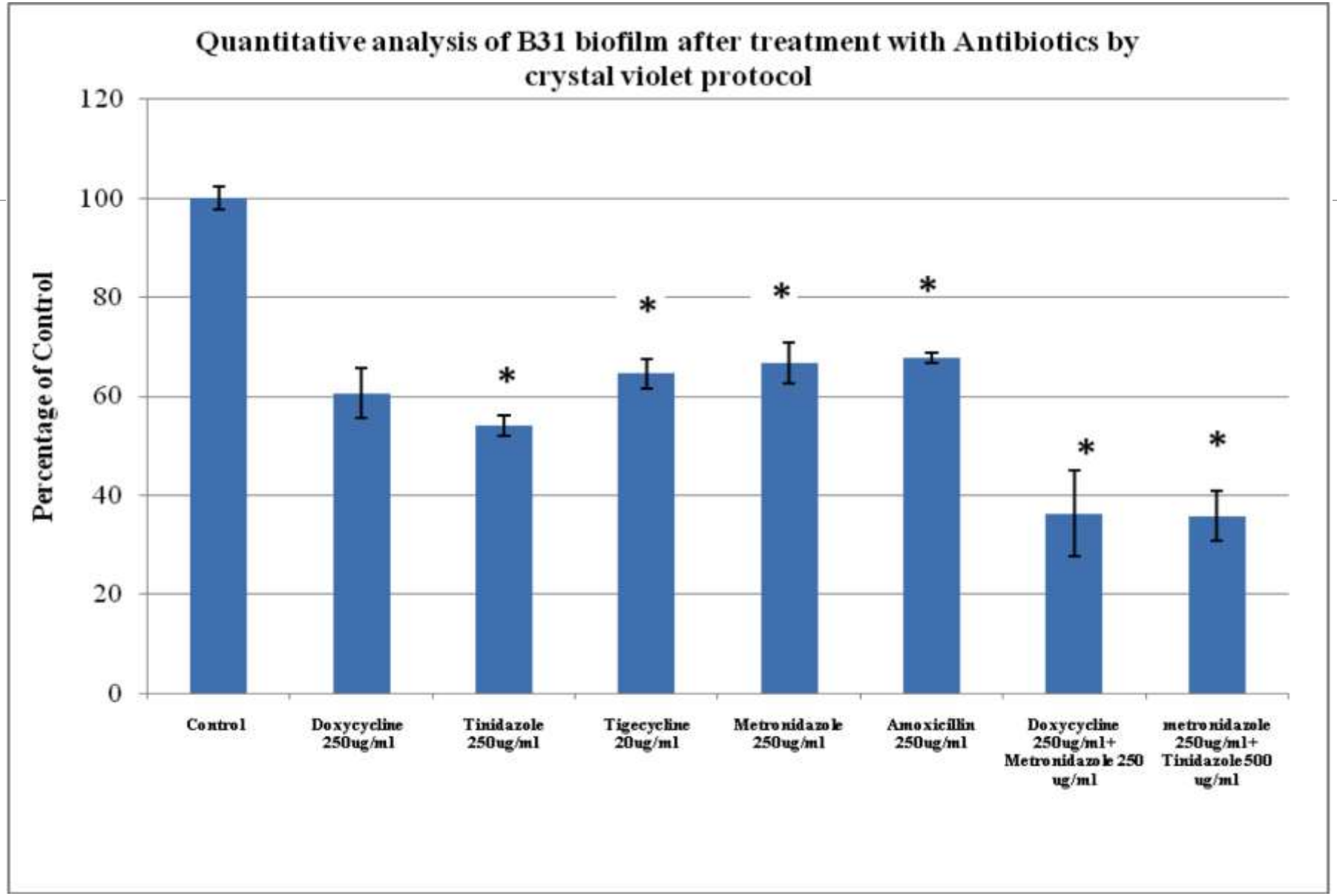
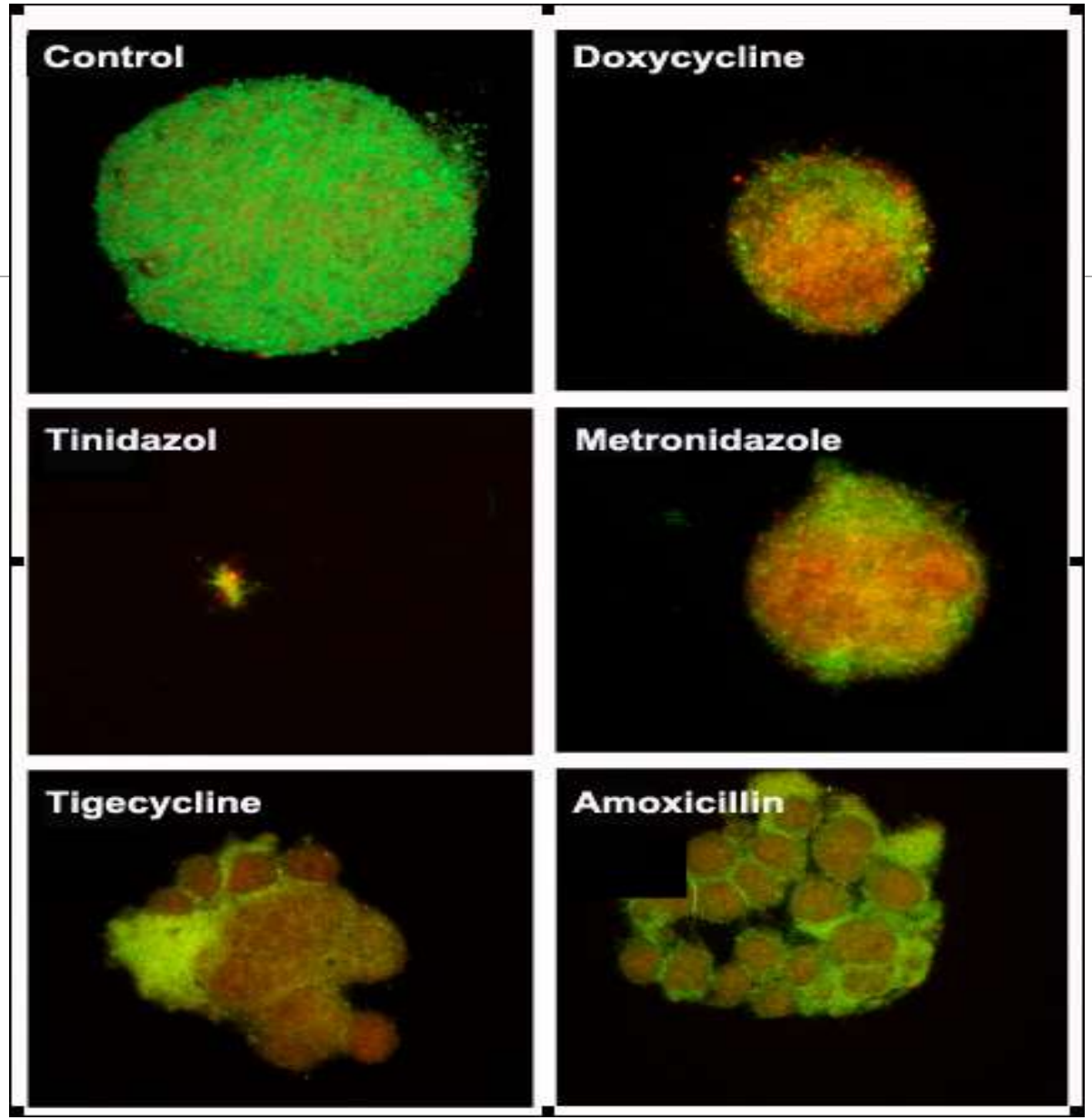
Kaur N, Sapi E 2012 unpublished data



Kaur N, Sapi E 2012 unpublished data



Red stain: Dead
Green stain: Viable

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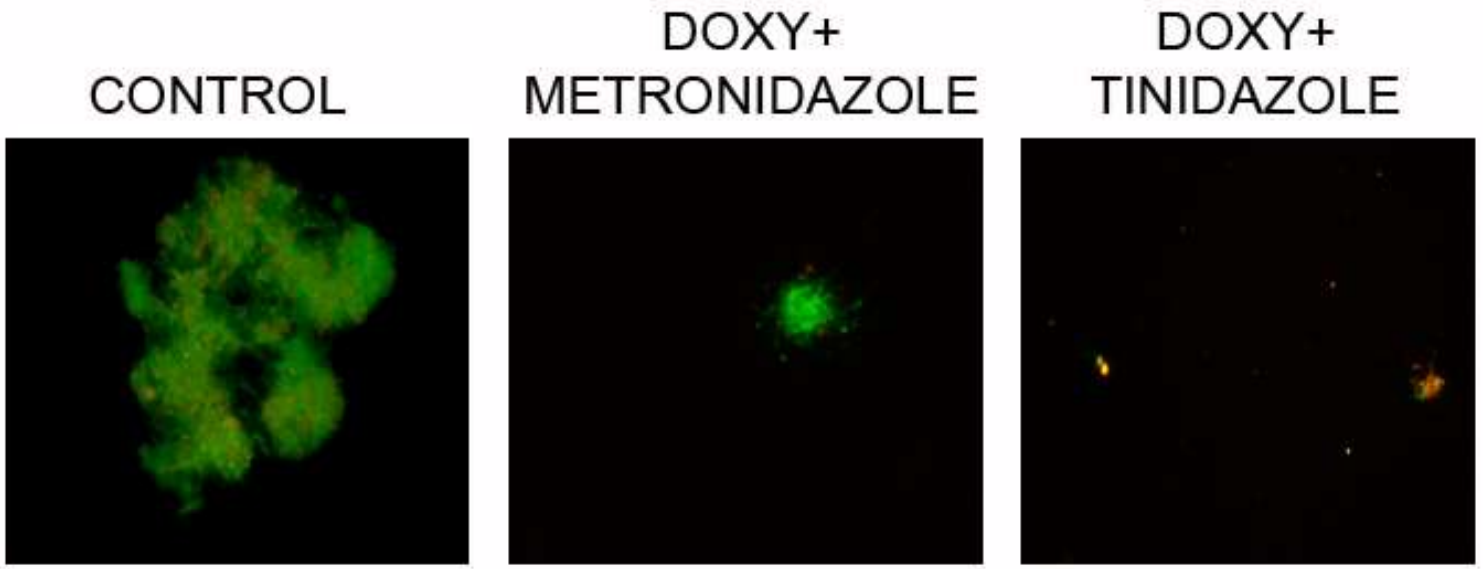
Effect of antibiotics on the aggregates of Borrelia measured BacLight staining Sapi E at al 2011

Combination treatment



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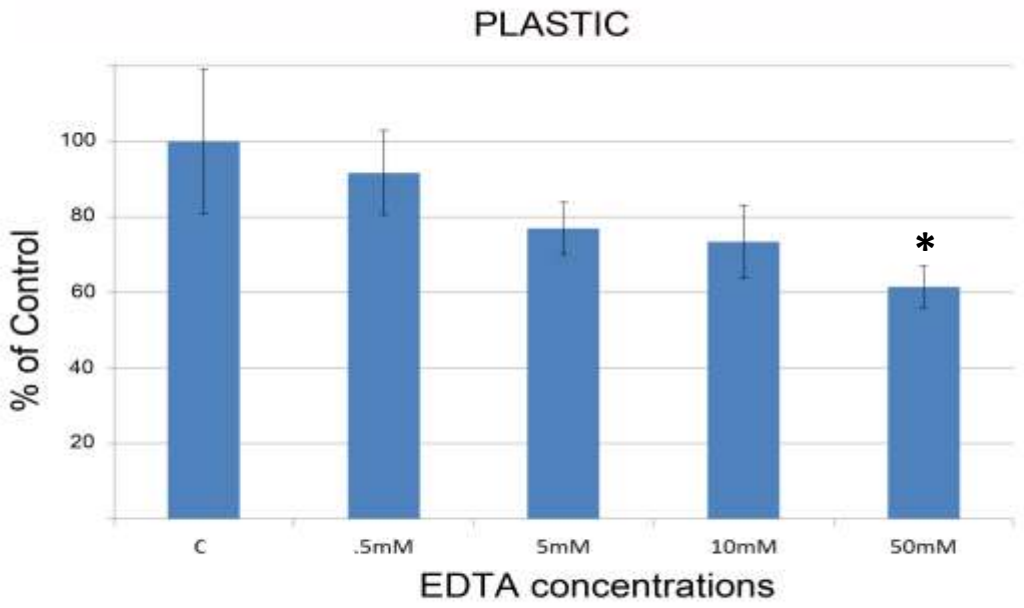
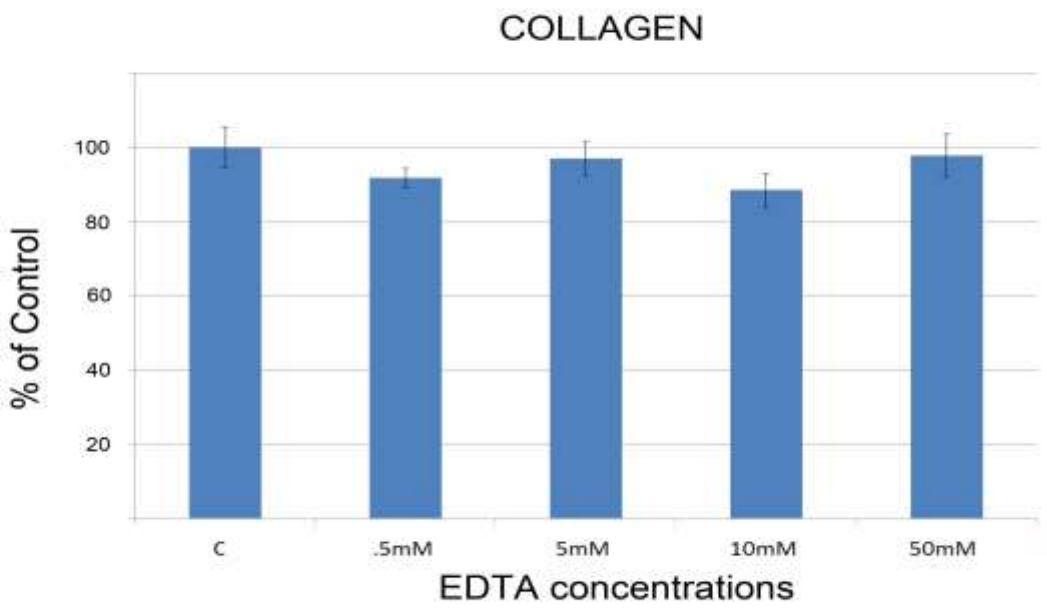
NEW HAVEN

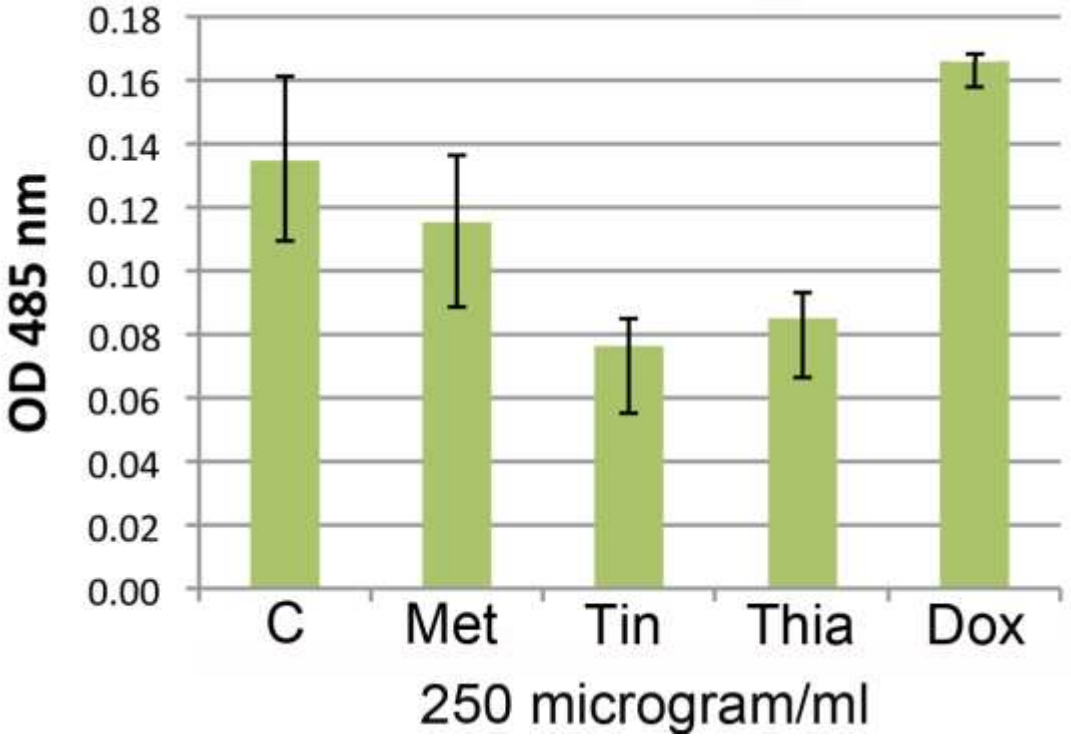
EDTA effect on *Borrelia* biofilm on plastic and on collagen - 48h

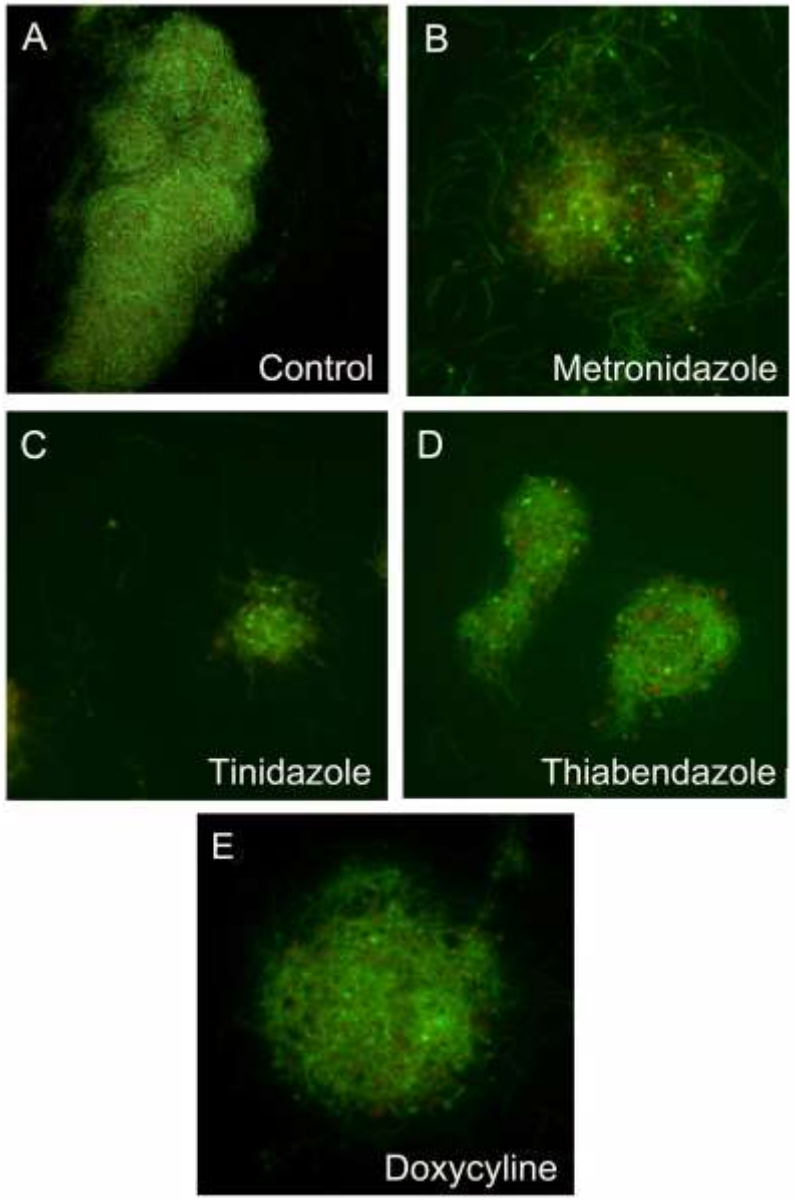




Phillips DS, Burugu D, Sapi E 2013 unpublished data

Effects of different *nitroimidazole* antibiotics on *Borrelia* biofilm (72h)





Theophilus PAS, Balu K Sapi E unpublished data 2013

Mixture of Enzymes?

Biofilm Defense

NEC proprietary enzyme blend

Pectinase

Glucoamylase

Serrazimes (endo/exo protease)

Nattozimes (endo/exo protease)

Beta-glucanase

Protease AM

Lipase

Protease 6.0

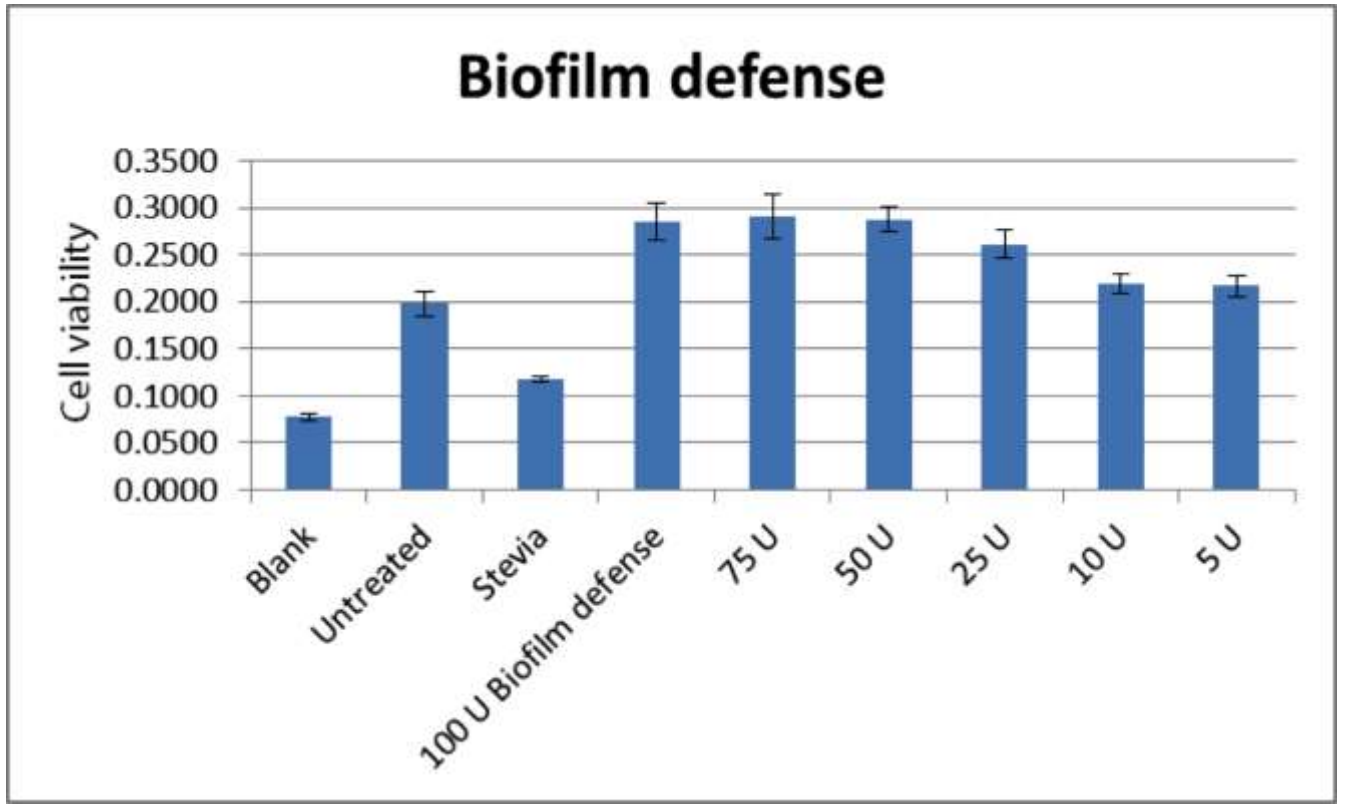
Alpha-galactosidase

Xylanase

Cellulase

Amylase

Invertase



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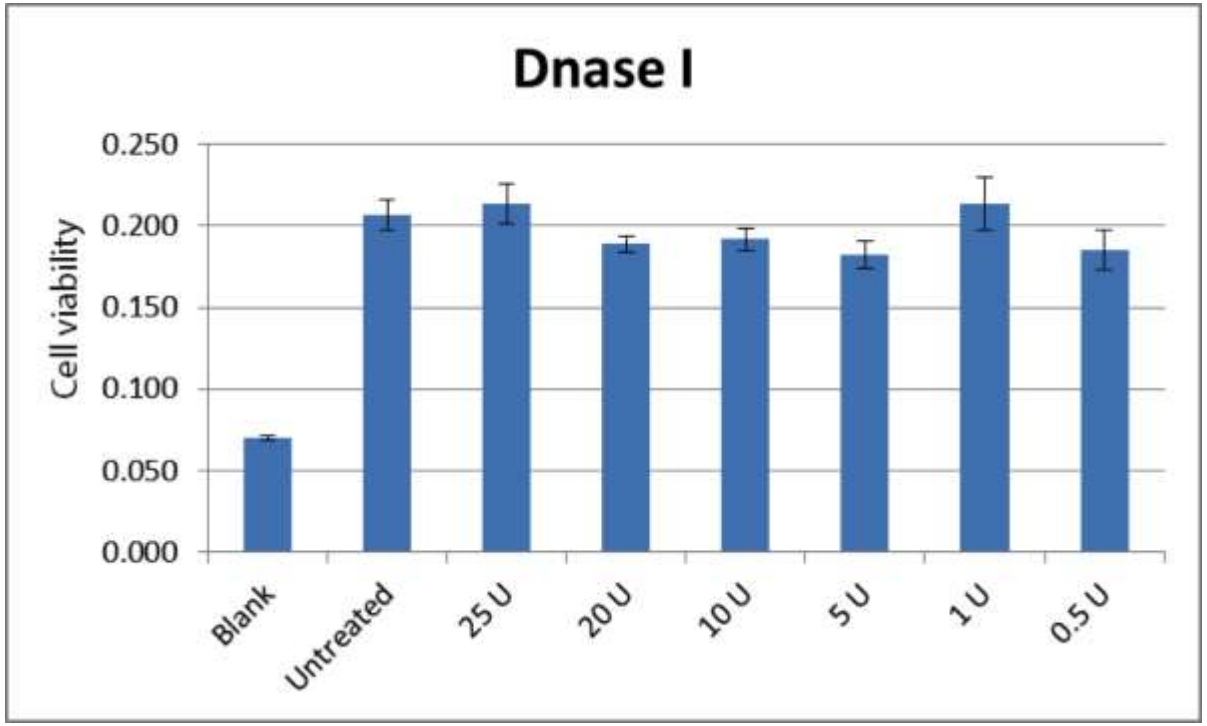
Victoria JM, Sapi E unpublished data 2014

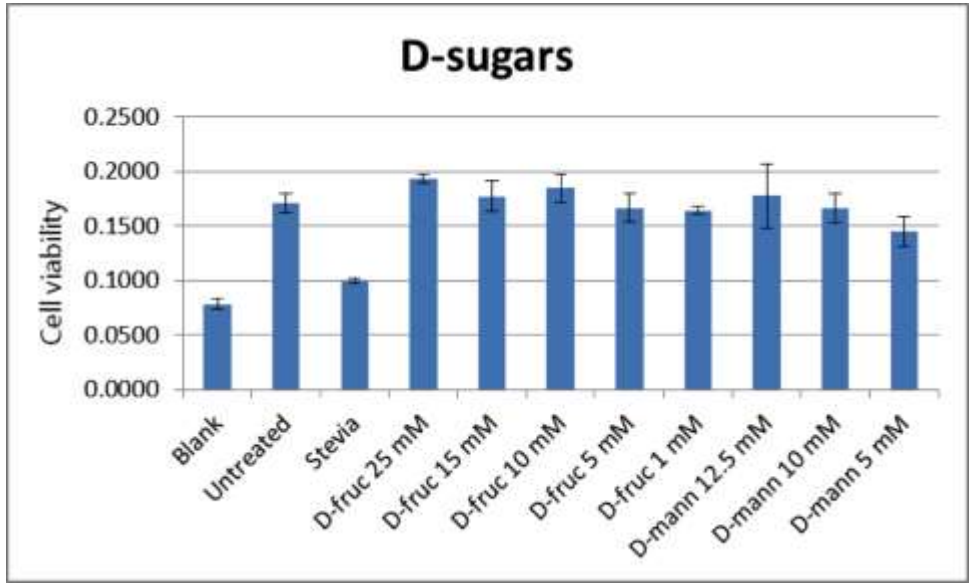
Degrading the extracellular DNA layer?

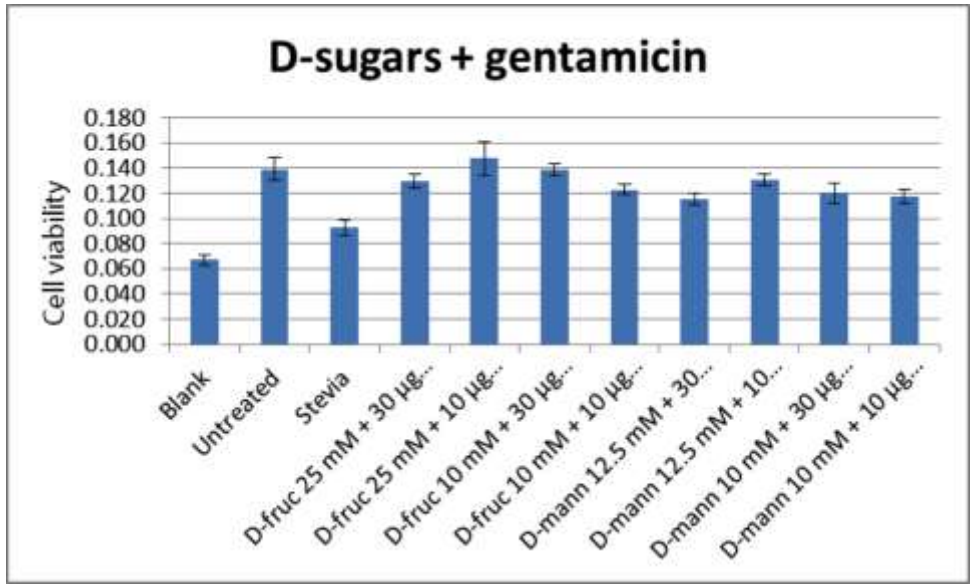


Victoria JM and Sapi E unpublished data 2013

Do we need a Trojan Horse?

Going after the persisters with sugar and aminoglycosides (Allison KR et al Nature 2011)





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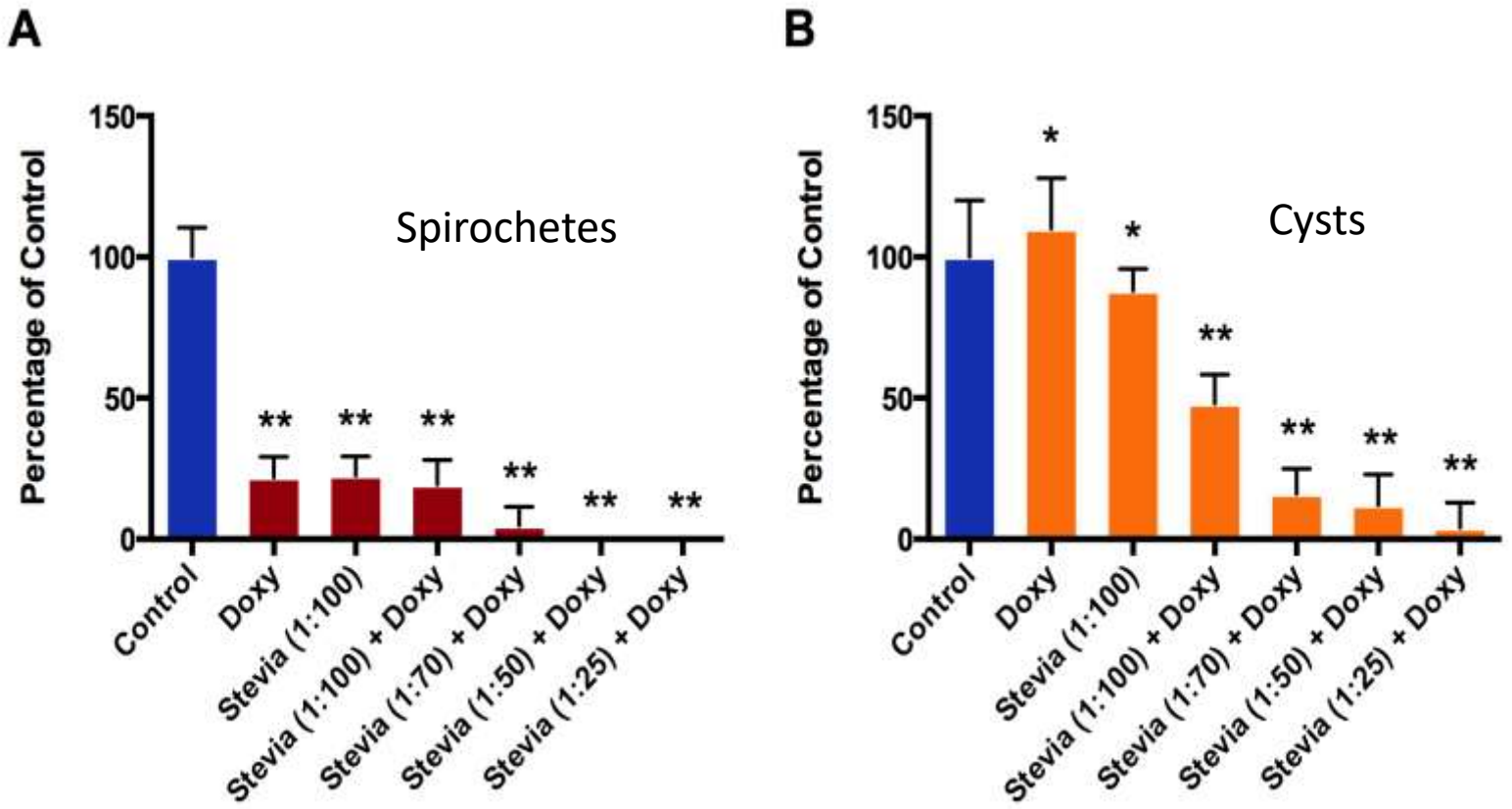
Victoria JM, Sapi E unpublished data 2014

Combining Stevia and Doxycyline?



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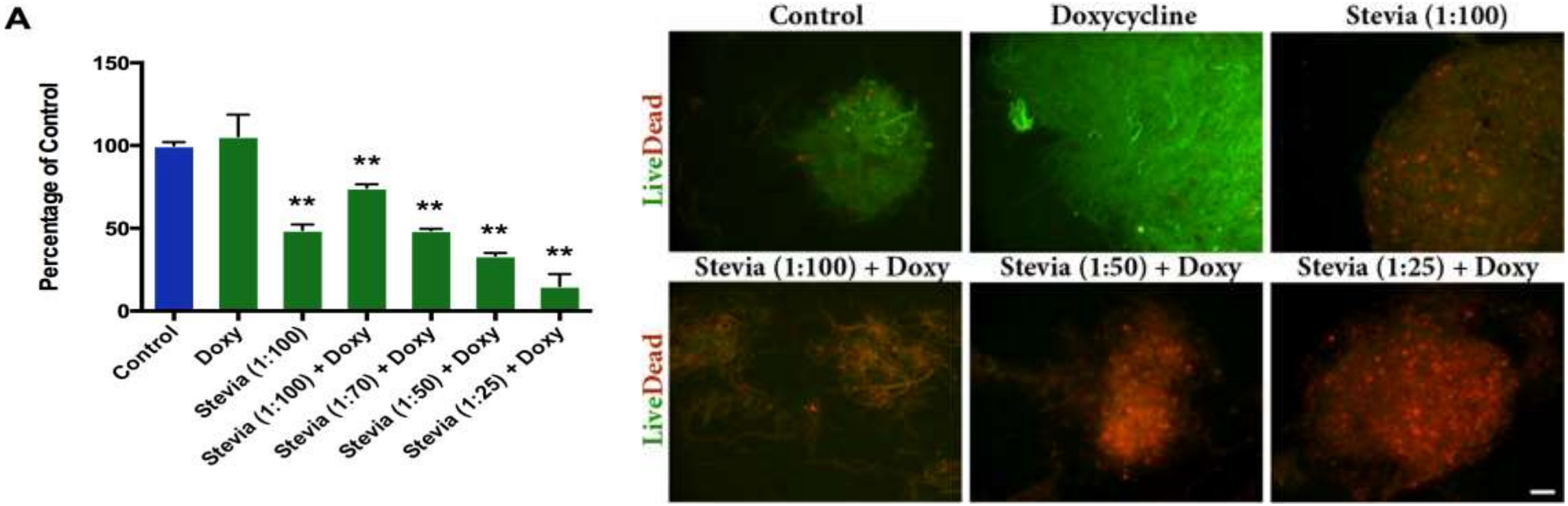
Theophilus PAS, Balu K Sapi E 2015

How about biofilm form?



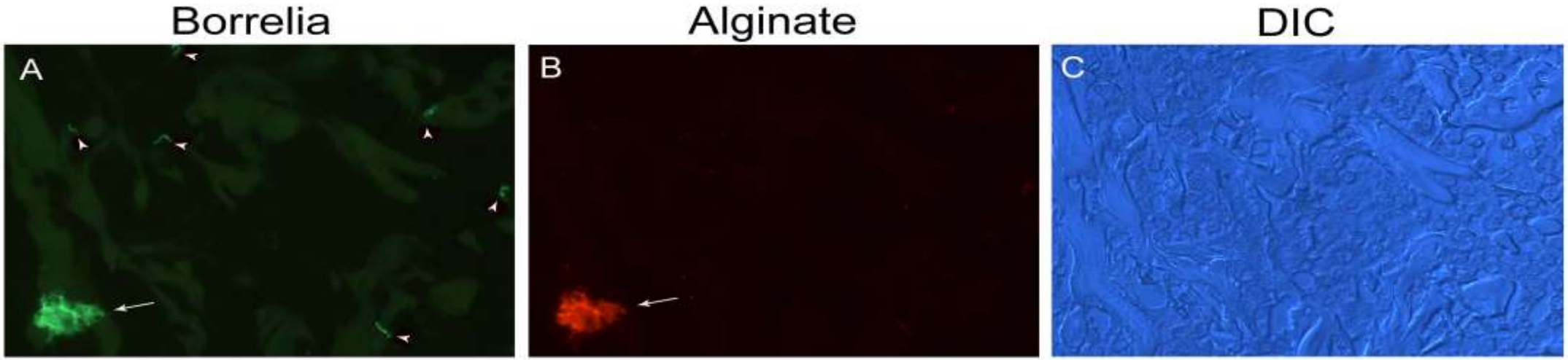
Theophilus PAS, Balu K Sapi E 2015

What is the next step?

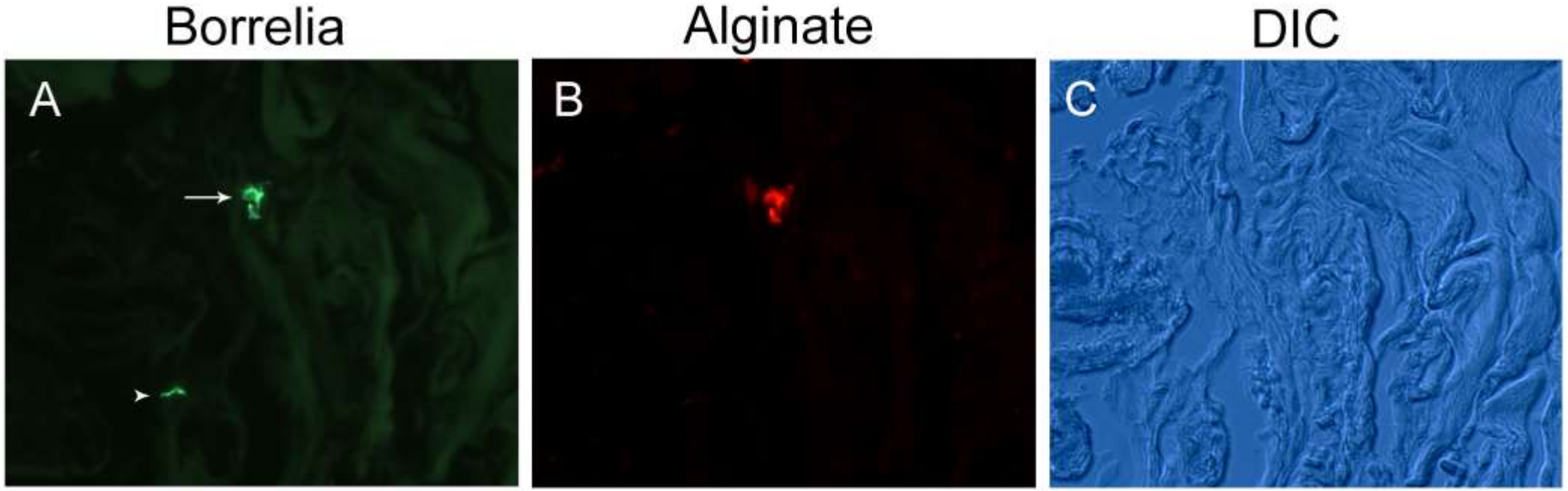
To demonstrate that *Borrelia* biofilms exist *in vivo*

- Skin biopsies from Borrelial lymphocytoma lesions *(Dr B Zelger)*

Double immunostaining: Borrelia and Alginate antibody – Skin biopsies



Additional findings

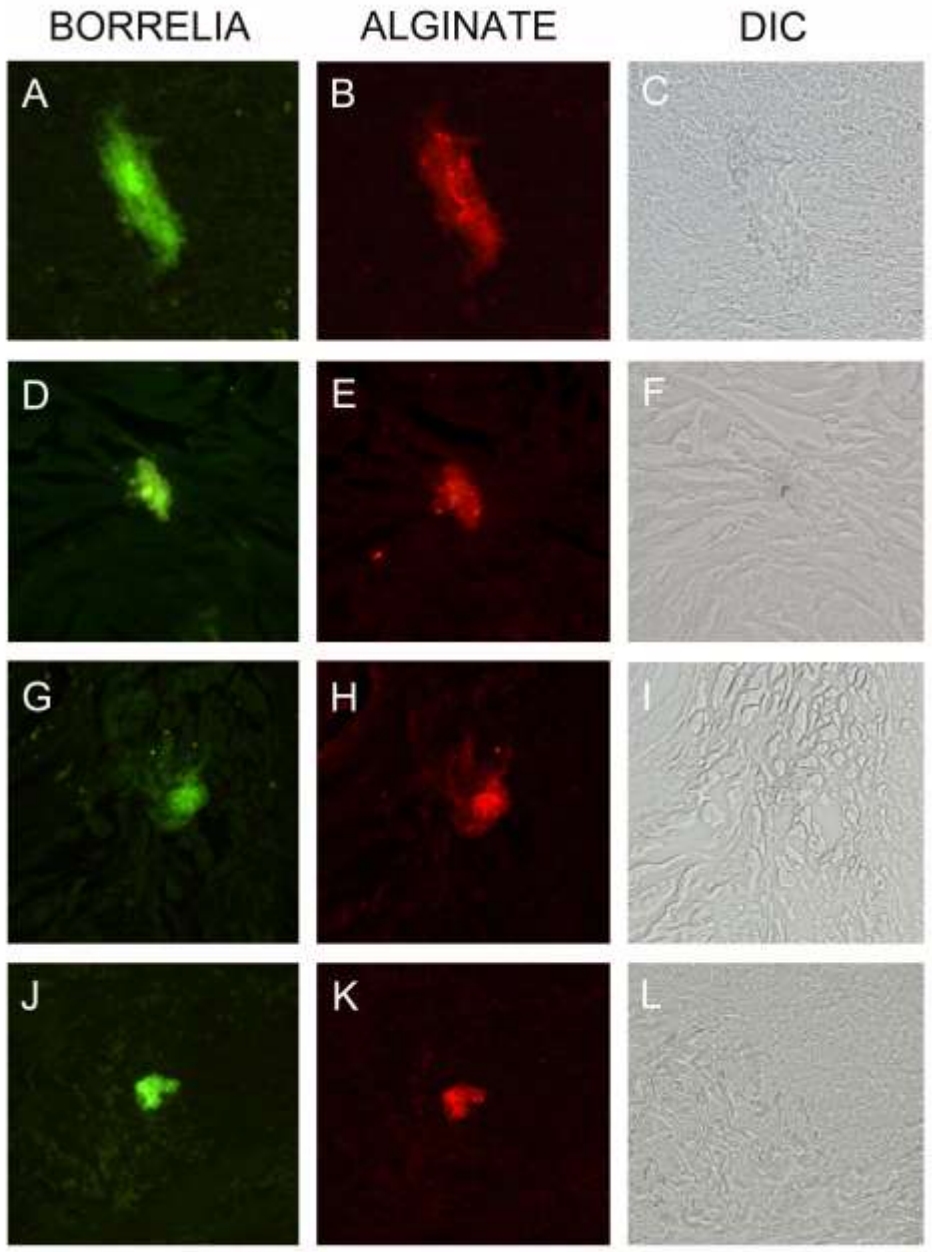


Immunohistochemical images of infecetd skin samles

Borrelia antigen (green staining) and alginate (red).

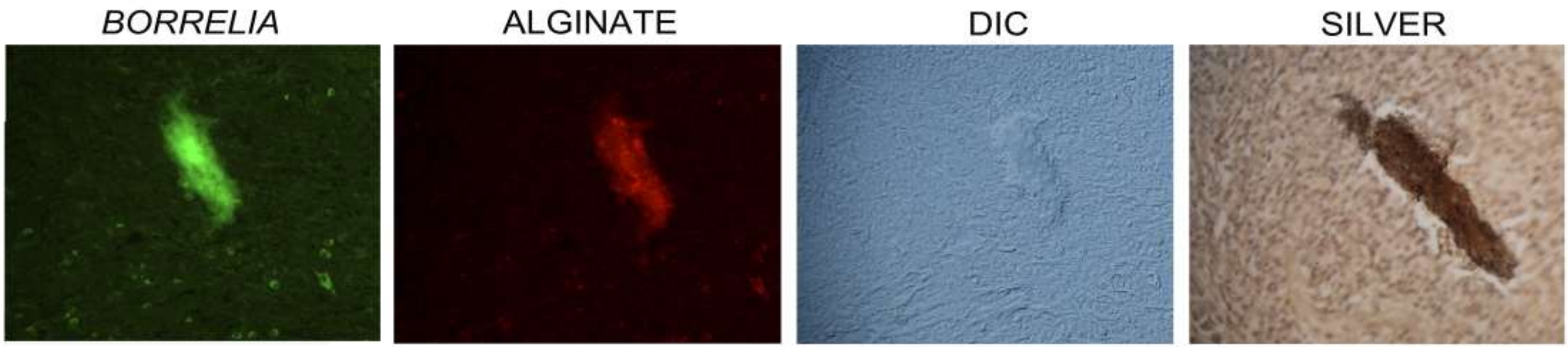
DIC= differential Interphase differential microscopy

All samples were confirmed with PCR/direct sequencing for Borrelia



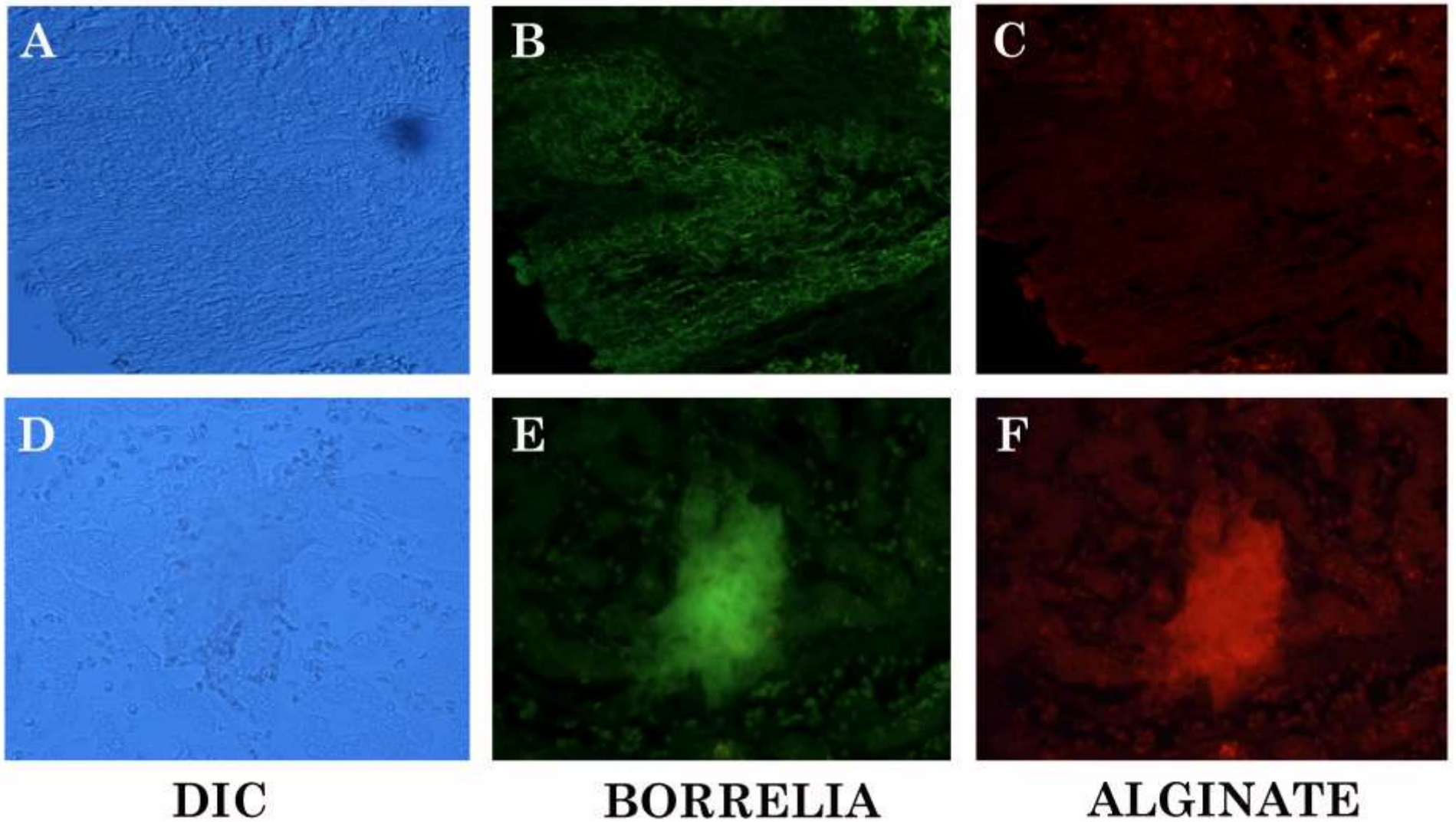
Sapi et al 2016

Borrelia biofilm with silver staining proof in infected skin tissue



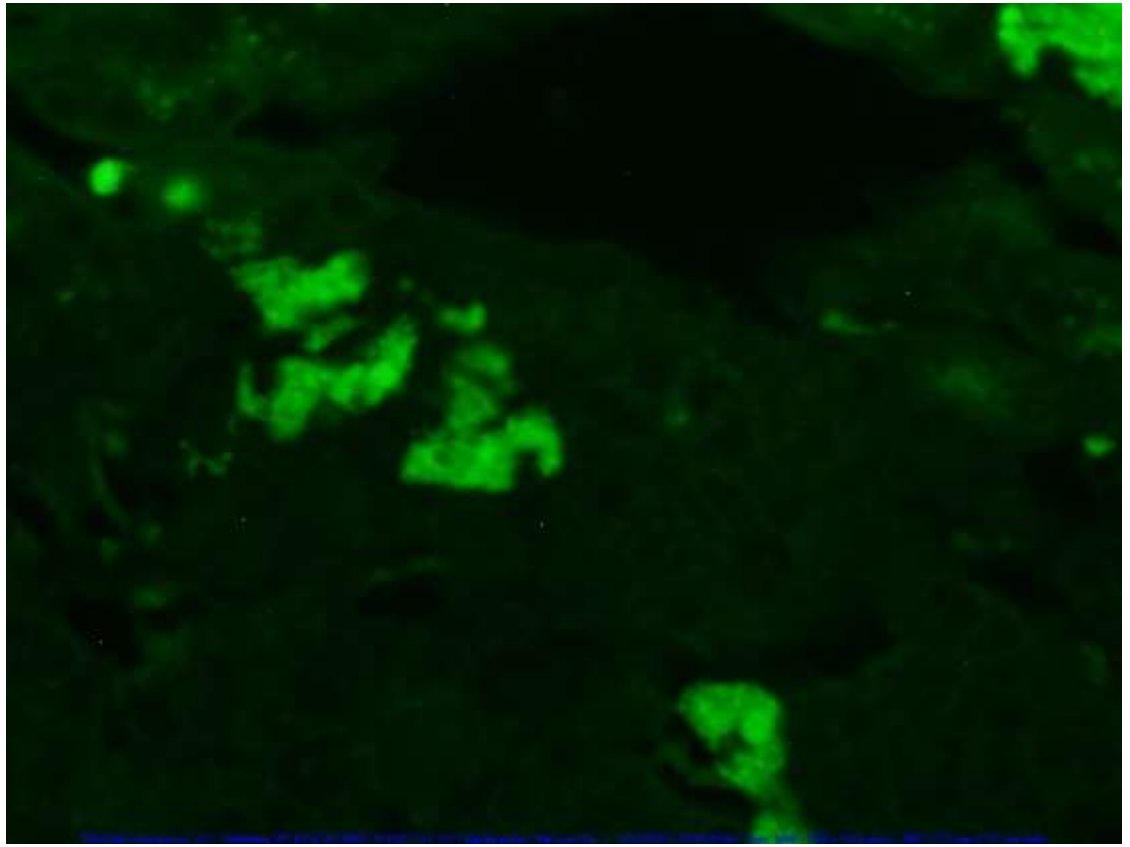
Sapi et al 2016

Where else can we find Borrelia biofilm in the body?

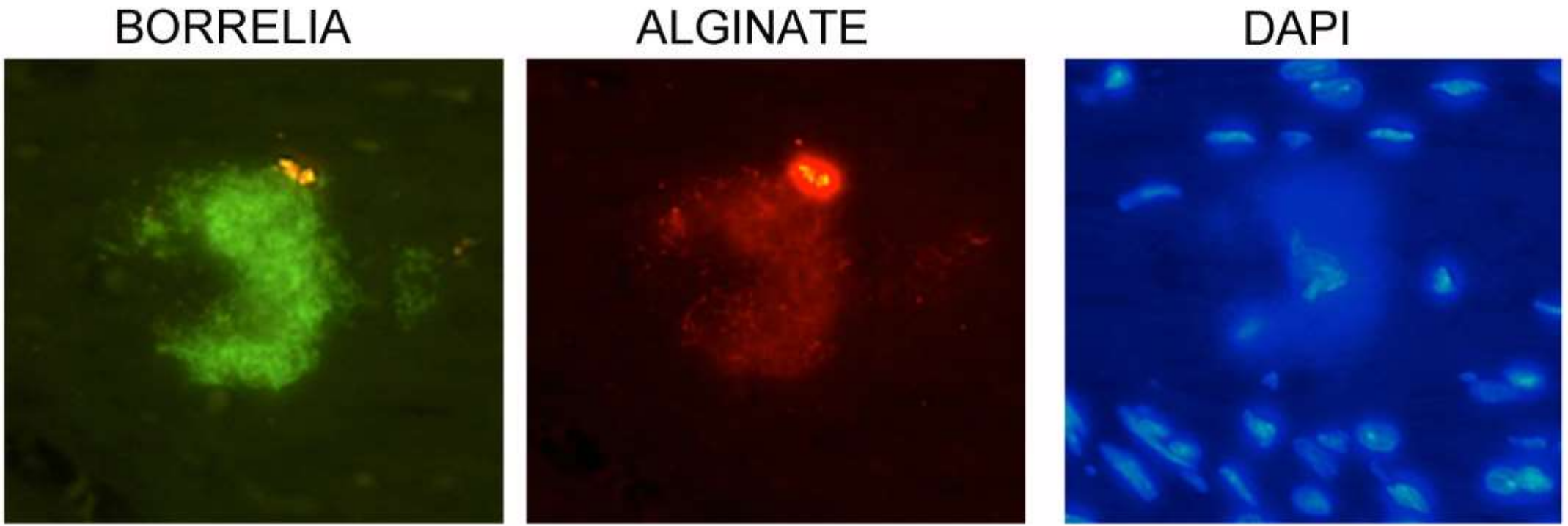


Balasubramanian K, Sapi E 2013 Unpublished data

Bb Infected Liver (MacDonald A, Middleveen M, 2014)



Mouse Heart Tissue infected by Borrelia



Summary

Borrelia burgdorferi does form a very organized biofilm *in vitro*

Major component is probably calcium alginate, but

polymer subunits remain to be identified

Biofilm provide a refuge from antimicrobial treatment

Preliminary results show potential *in vivo* Borrelia biofilm in infected skin tissues as well other tissues

The new atomic force microscope
UNH Lyme disease research group

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Dr. Michael Rossi and Dr. Roman Zajac at the Department of Biology and Environmental Science (UNH) for additional support and funds

Dr. Alan MacDonald for his never-ending support

Question?

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