Female Adult with Chronic Lyme Borreliosis, Oslo Norway – Blood study with Borrelia specific DNA probes using Fluorescent in Situ Dna Hybridization [FISH method] to detect spirochetes of Burgdorferi Borrelia (sl group) and Miyamotoi borrelia Specimen obtained October 27, 2015- All Controls for FISH method are Acceptable: Respectfully submitted, Alan B. MacDonald, MD Fellow, College of American Pathologists

Research Methodology:

EDTA anticoagulated peripheral blood (purple top tube) routine blood smears are prepared [either Wedge smears or Buffy Coat Smears]. Smears are Air Dried, labelled with patient identifiers [a code may be used for anonymity at patient discretion]

10 glass slide blood smear preparations are placed in plastic Slide mailer container, then wrapped in Bubble wrap and mailed to Researcher. Upon receipt- Blood smears are fixed in 100% denatured ethanol of 10 minutes and are dried thoroughly.

10 microliter of the Working dilution of each molecular Beacon are placed on each slide and then 500 microliters of 100 % DMF [Dimethylformamide] A plastic rectangle of Ziploc® freezer Bag is trimmed to the size of a 24 by 50 mm glass coverslip, and is placed over the spotted area of the Molecular Beacon drop on the slide. Gentle pressure with a plastic spatula removes all air bubbles beneath the plastic rectangle.

Each slide is heated to 90 degrees C. Careful monitoring of the slide surface temperature is constantly monitored by a Laser Thermoprobe. The slide is placed on a 72-degree surface for 30 minutes. The temperature of the slide surface is carefully monitored with the Thermoprobe. After 30 minutes the slides with plastic rectangle covers are allowed to return to 24 degrees C.

The plastic rectangles are carefully removed to avoid maceration of the blood smear. Each slide is washed by immersion in 0.9% normal buffered saline solution ph. 7.0. The washed slides are allowed to fully dry in a dust free environment. One drop of 100% glycerol is applied to the Hybridized slides. A glass coverslips 24 x 50 mm [0,13-0.17 mm thickness] is gently lowered onto the slide. Air bubbles are expressed from the edge of the coverslip by compression between two paper towels.

FISH hybridized slides are examined with an Epifluorescent Microscope outfitted with filter barrier Cubes for FITC [green color fluor], Cy5 [red color fluor], and Cy3 [yellow color fluor] Fluorochromes.

Photographs are taken of fields of view which demonstrate Fluorescence with FITC or Cy5 fluorochromes

Molecular Beacon type DNA Probes:

1. 22 NUCLEOTIDE Sequence - from BBO 0147 on the chromosome [Flagellin B of ***B. burgdorferi***.]: Fluorescein label (FITC) – Green color when Bound to target DNA of mRNA- AT 100% MATCH – Mandatory perfect match]

2. 30 NUCLEOTIDE Sequence – from Flagellin B of ***Miyamotoi Borrelia*** on chromosome. {RED color when bound - Cy5 label}: Bound to target DNA of mRNA- AT 100% MATCH – Mandatory perfect match]
3. 30 NUCLEOTIDE Sequence – From GlpQ of ***miyamotoi borrelia*** on the chromosome. [Yellow color when bound- Cy3 label]: Bound to target DNA of mRNA- 100% MATCH – Mandatory perfect match]

Controls for DNA probe Hybridizations:

A: Melting curve Analysis: Beacon is incubated with its complement nucleotide sequence: A range of incubation temperatures – examined – and the magnitude of fluorescence plotted for each temperature. Binding of Beacon to complementary nucleotide: measured for each degree increase from room temperature to 90 degrees C. An optimal hybridization "annealing" temperature is determined for each Molecular Beacon.

B. BLASTn supercomputer searches in NCBI GenBank Websites; Rank order of homologies In nucleotide sequences from all life forms on planet earth is displayed. Only ***borrelia*** species are found to have 100% sequence matches with Molecular Beacon Probes. Borrelia burgdorferi gene bb0 0740 {inner cell membrane gene of ***Burgdorferi Borrelia group (sl)*** shows 100% search matches in BLASTn Searches. Flagellin B Miyamotoi: only ***B***. ***miyamotoi*** strains show 100% match results in BLASTn. GlpQ ***Miyamotoi***: Only B. ***miyamotoi*** strains show 100% match in BLASTn searches. ***B. Burgdorferi***: Only species within the group of B. ***Burgdorferi*** (sl) [sensu lato] show 100% matches with Beacon Sequence for bbo 0740 ***Burgd.***

C. Tick Squash preparations: Use of Beacons for ***burgdorferi*** Flagellin B, and ***miyamotoi borrelia*** Flagellin B demonstrates the detection of ***Burgdorferi borrelia*** in a cohort of samples and rare ticks carrying ***Miyamotoi borrelia.*** No Ticks with simultaneous carriage of both ***borrelia*** species are encountered in a small sample of ***Ixodid*** ticks examined.

D. Pure cultures of the B31 strain of ***Borrelia burgdorferi*** [ATCC 35210] hybridize with the ***B. burgdorferi*** flagellin B Molecular Beacon and with the bbo 0740 molecular beacon., ***Burgd.*** FlaB) but do not hybridize with the ***miyamotoi borrelia*** molecular beacons (Miya. Fla or GlpQ).

Fluorescence signals upon successful in Situ DNA Hybridization of Molecular Beacon DNA probes

{as detailed above} to demonstrate conclusive evidence of

A. ***borrelia*** living microbes and

B. Species of ***borrelia*** hybridizing in situ with specific DNA probes.

See Images:

Controls: Molecular Beacons: Color Images below

From Tick squash preparations (Miyamotoi and *burgdorferi borrelia*, and from pure cultures of B31 strain of *Burgdorferi Borrelia* grown in vitro.

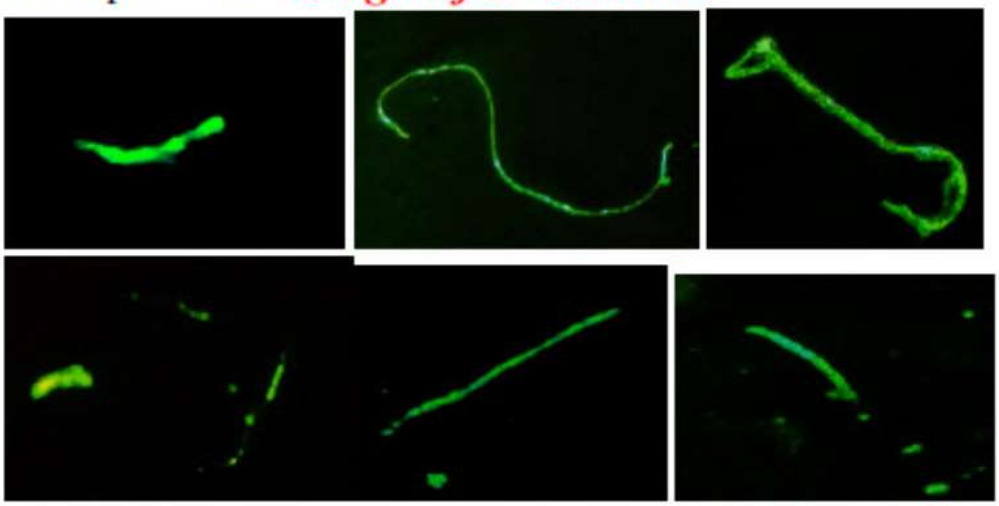
FITC label – DNA Beacon probe for Burgdorferi borrelia –gene bbo 0740

Cy5 label – DNA Beacon probe for Miyamotoi borrelia – Fla B

Cy3 label - DNA Beacon probe for Miyamotoi borrelia – GlpQ {this DNA probe was not used in this Case study because the supply was temporarily used up in other experiments}

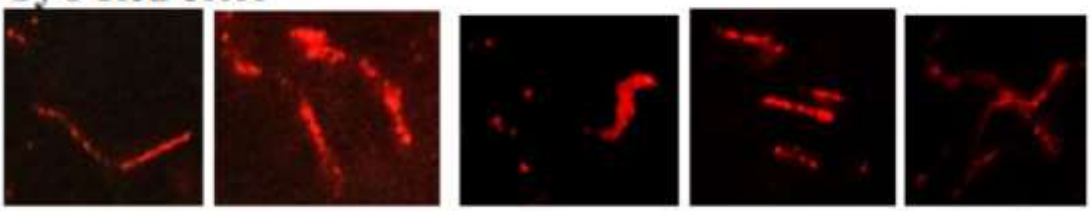
Female Adult with Chronic Lyme Borreliosis, Oslo Norway – Blood study with Borrelia specific DNA probes using
Fluorescent in Situ Dna Hybridization [FISH method] to detect spirochetes of Burgdorferi Borrelia (sl group) and Miyamotoi borrelia
Specimen obtained October 27, 2015- All Controls for FISH method are Acceptable: Respectfully submitted, Alan B. MacDonald, MD
Fellow, College of American Pathologists

Green Color Images: Flagellin B (FlaB probe 147) and Inner Cell membrane probe 740 Fluorescent In Situ DNA Hybridization [FISH METHOD] using **FITC (GREEN color)** DNA probes to ***Burgdorferi Borrelia***

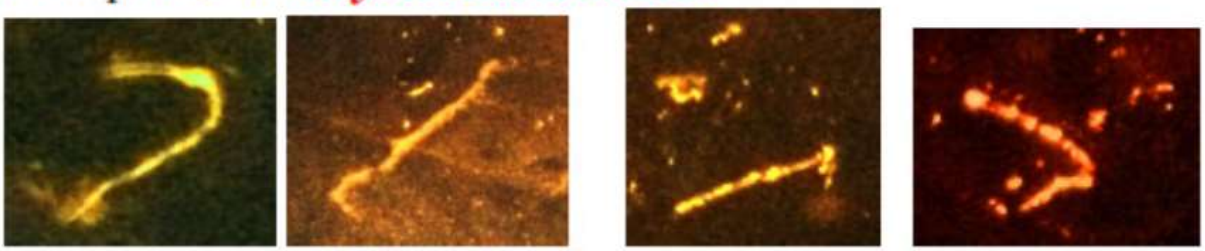


Female Adult with Chronic Lyme Borreliosis, Oslo Norway – Blood study with Borrelia specific DNA probes using
Fluorescent in Situ Dna Hybridization [FISH method] to detect spirochetes of Burgdorferi Borrelia (sl group) and Miyamotoi borrelia
Specimen obtained October 27, 2015- All Controls for FISH method are Acceptable: Respectfully submitted, Alan B. MacDonald, MD
Fellow, College of American Pathologists

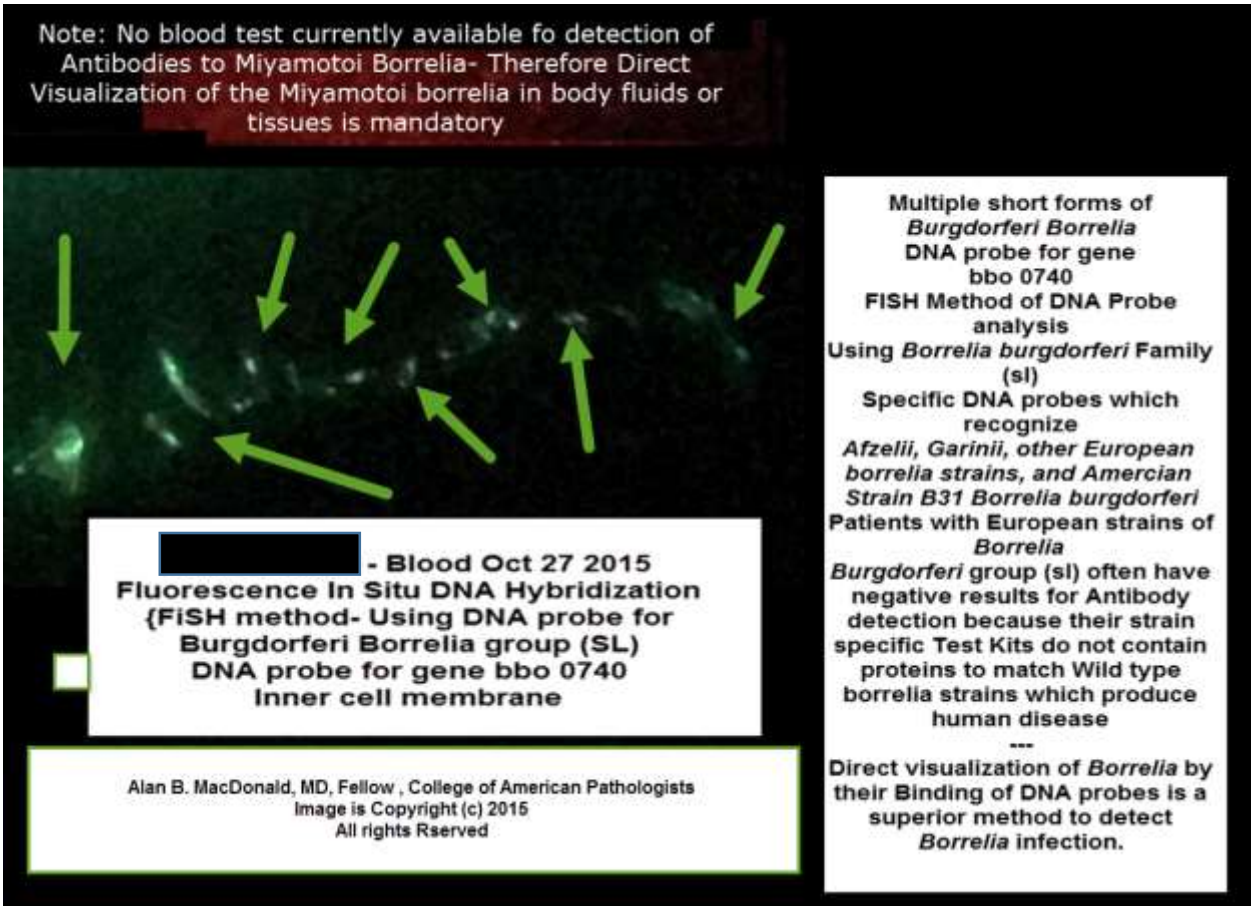
Red Color Images: Gene Flagellin B of Miyamotoi Borrelia: Cy 5 Red color



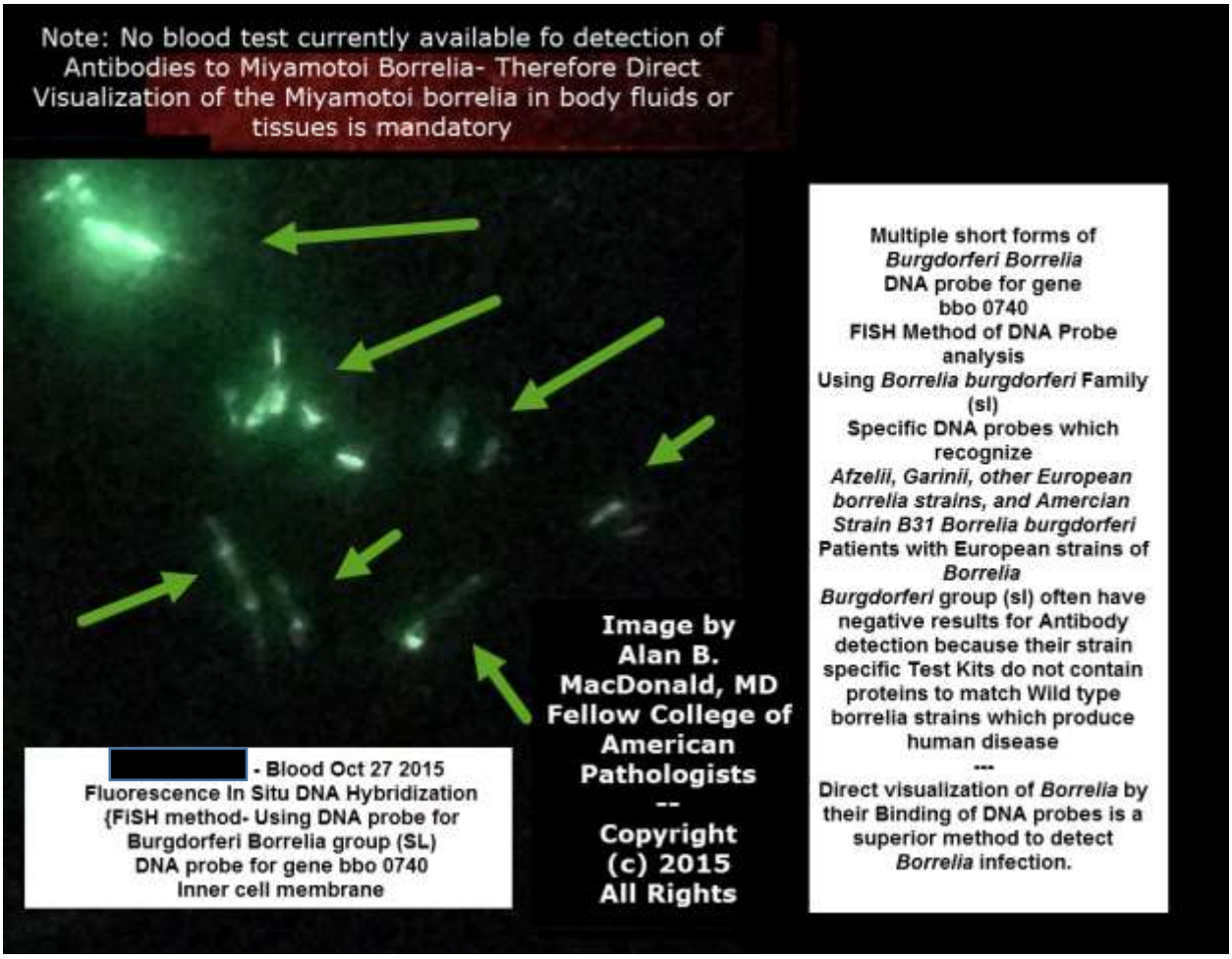
Yellow Color Images: Gene **GlpQ of** *Miyamotoi Borrelia* and ***Relapsing Fever Borrelia*** Fluorescent In Situ DNA Hybridization [FISH METHOD] Using **Cy 3 (Yellow color)** DNAprobes to ***Miyamotoi Borrelia***



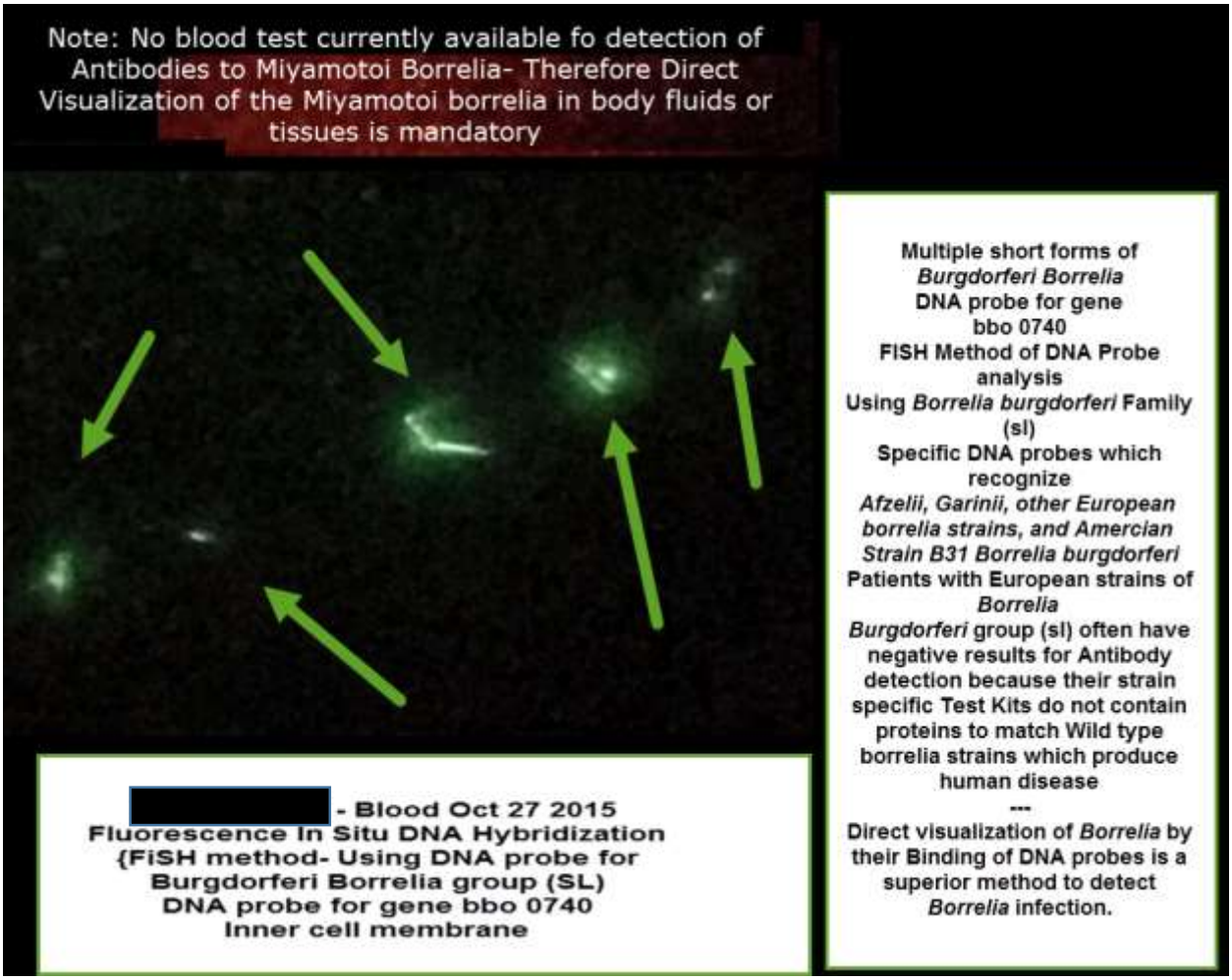
Patient Results: Blood Smear examination: October 27, 2015



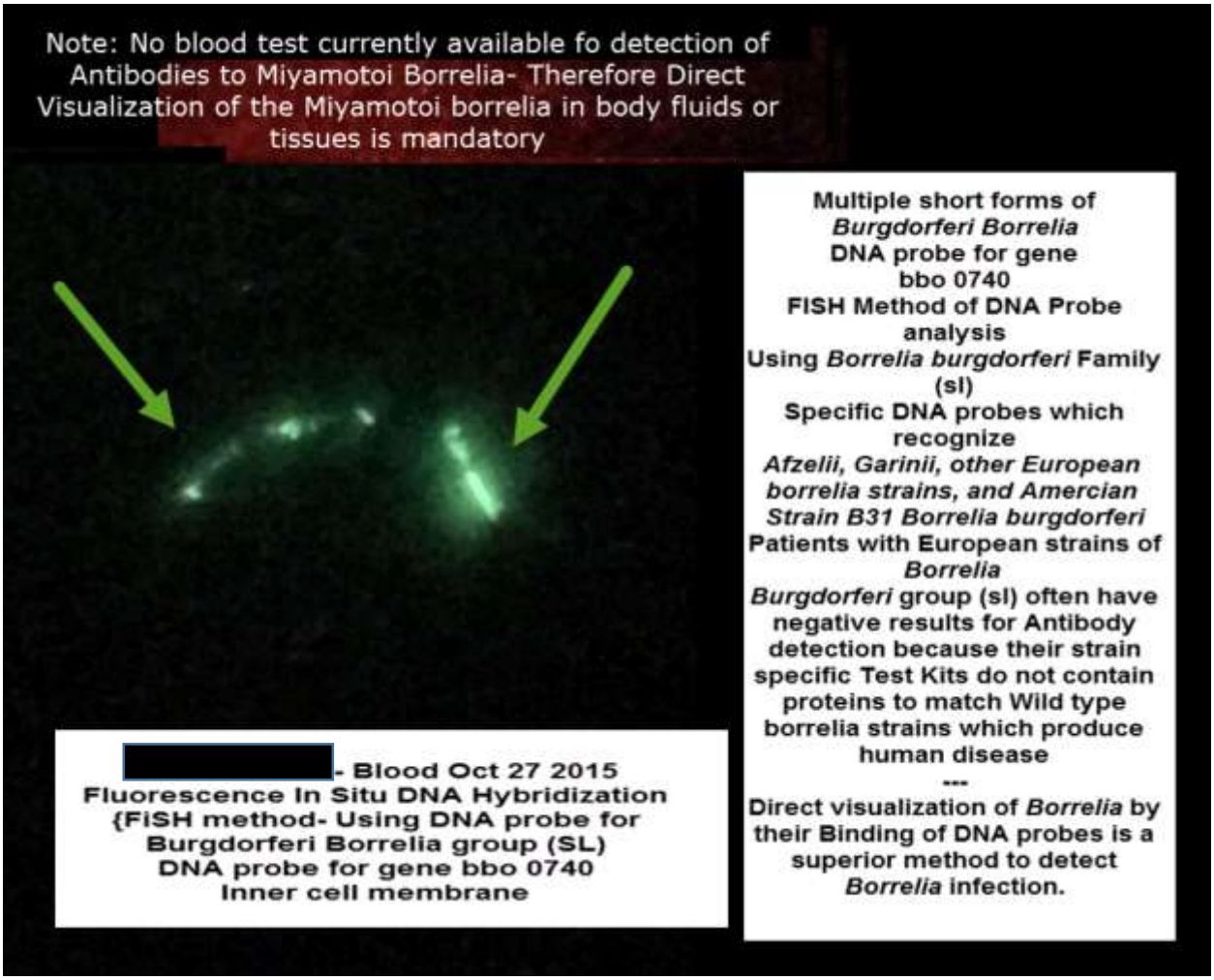
Female Adult with Chronic Lyme Borreliosis, Oslo Norway – Blood study with Borrelia specific DNA probes using
Fluorescent in Situ Dna Hybridization [FISH method] to detect spirochetes of Burgdorferi Borrelia (sl group) and Miyamotoi borrelia
Specimen obtained October 27, 2015- All Controls for FISH method are Acceptable: Respectfully submitted, Alan B. MacDonald, MD
Fellow, College of American Pathologists



Female Adult with Chronic Lyme Borreliosis, Oslo Norway – Blood study with Borrelia specific DNA probes using
Fluorescent in Situ Dna Hybridization [FISH method] to detect spirochetes of Burgdorferi Borrelia (sl group) and Miyamotoi borrelia
Specimen obtained October 27, 2015- All Controls for FISH method are Acceptable: Respectfully submitted, Alan B. MacDonald, MD
Fellow, College of American Pathologists

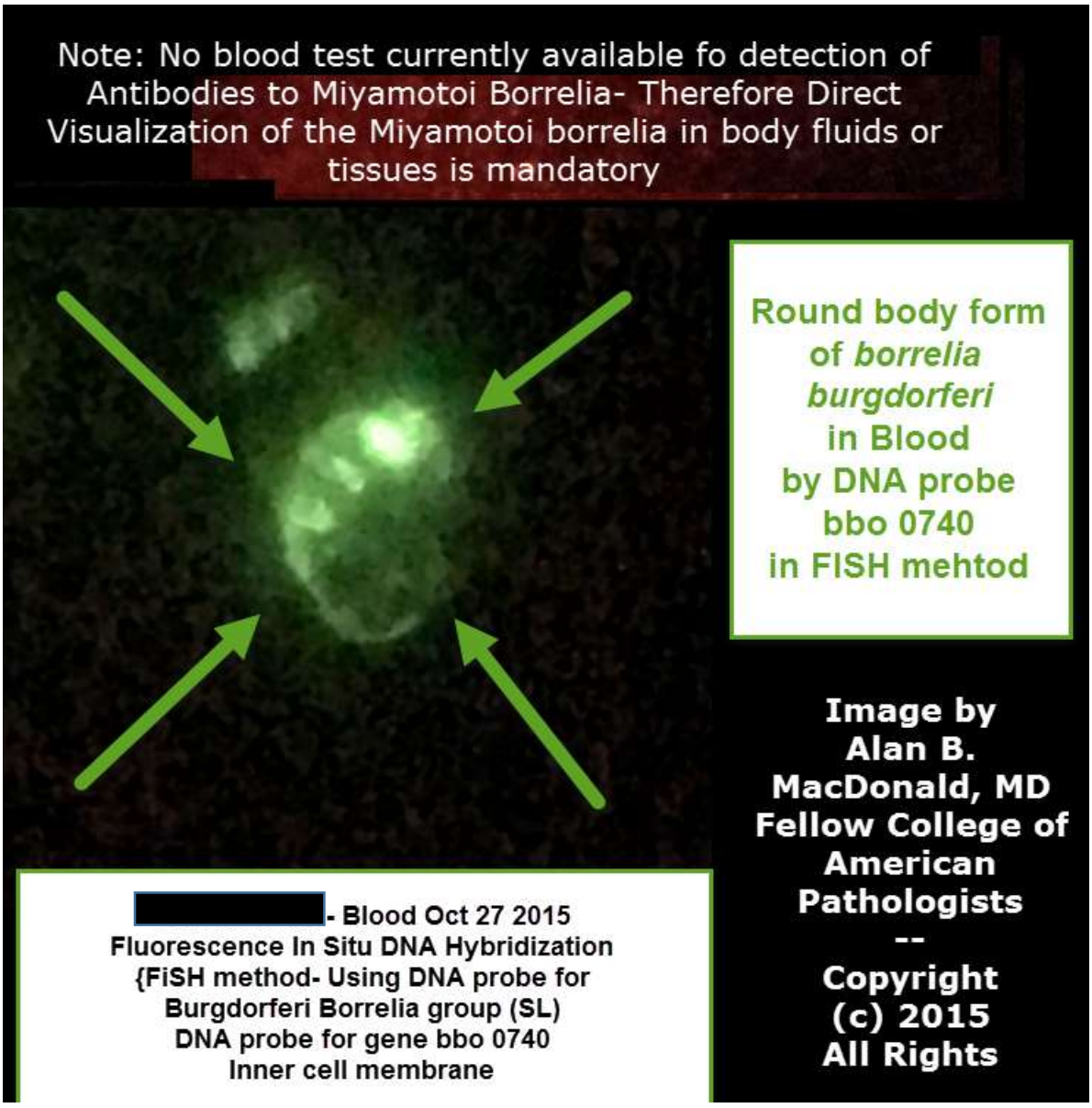


Female Adult with Chronic Lyme Borreliosis, Oslo Norway – Blood study with Borrelia specific DNA probes using
Fluorescent in Situ Dna Hybridization [FISH method] to detect spirochetes of Burgdorferi Borrelia (sl group) and Miyamotoi borrelia
Specimen obtained October 27, 2015- All Controls for FISH method are Acceptable: Respectfully submitted, Alan B. MacDonald, MD
Fellow, College of American Pathologists

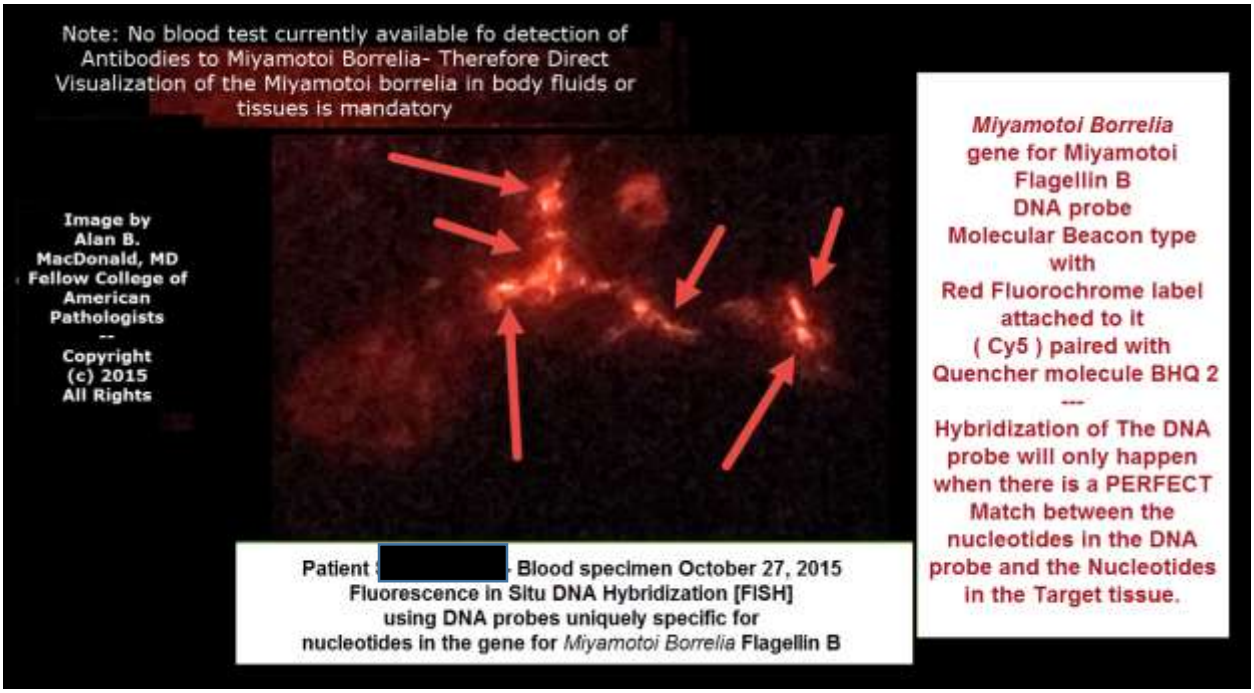


Female Adult with Chronic Lyme Borreliosis, Oslo Norway – Blood study with Borrelia specific DNA probes using
Fluorescent in Situ Dna Hybridization [FISH method] to detect spirochetes of Burgdorferi Borrelia (sl group) and Miyamotoi borrelia
Specimen obtained October 27, 2015- All Controls for FISH method are Acceptable: Respectfully submitted, Alan B. MacDonald, MD
Fellow, College of American Pathologists

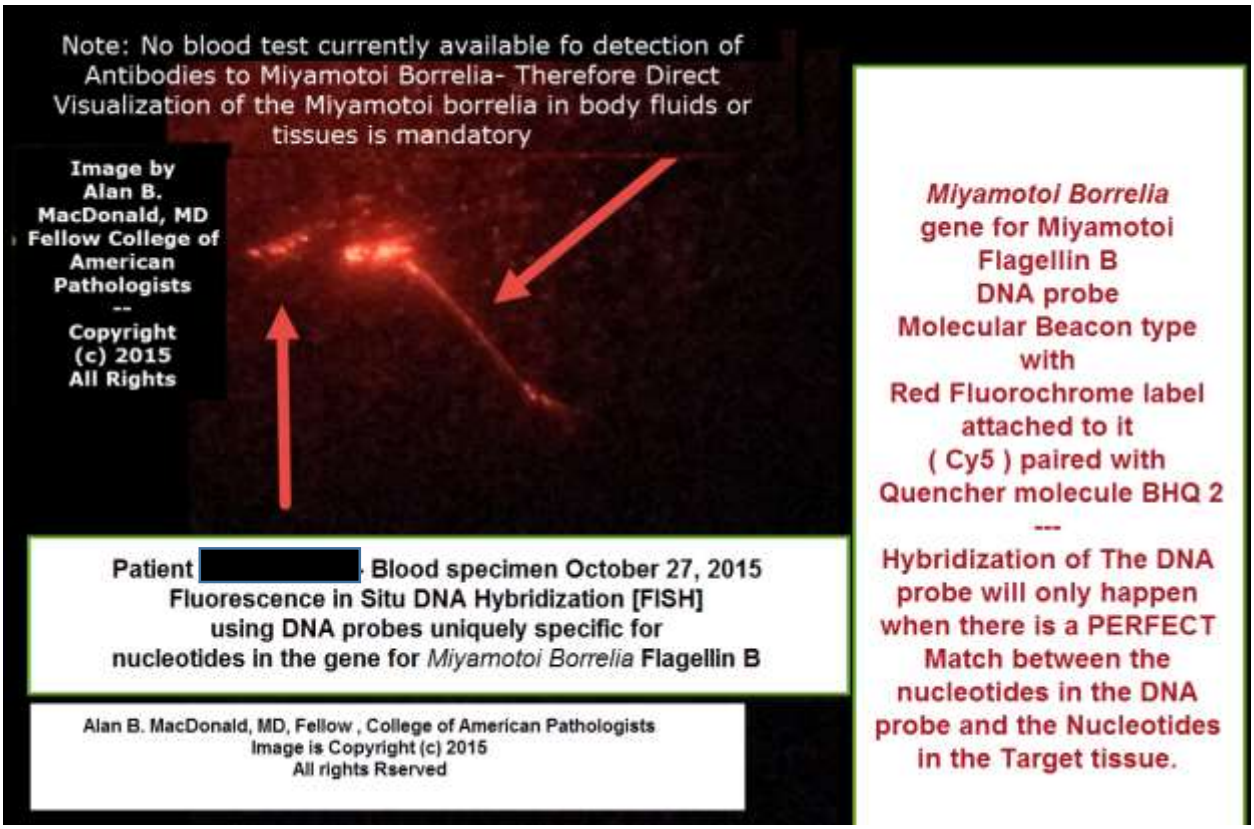
Female Adult with Chronic Lyme Borreliosis, Oslo Norway – Blood study with Borrelia specific DNA probes using
Fluorescent in Situ Dna Hybridization [FISH method] to detect spirochetes of Burgdorferi Borrelia (sl group) and Miyamotoi borrelia
Specimen obtained October 27, 2015- All Controls for FISH method are Acceptable: Respectfully submitted, Alan B. MacDonald, MD
Fellow, College of American Pathologists



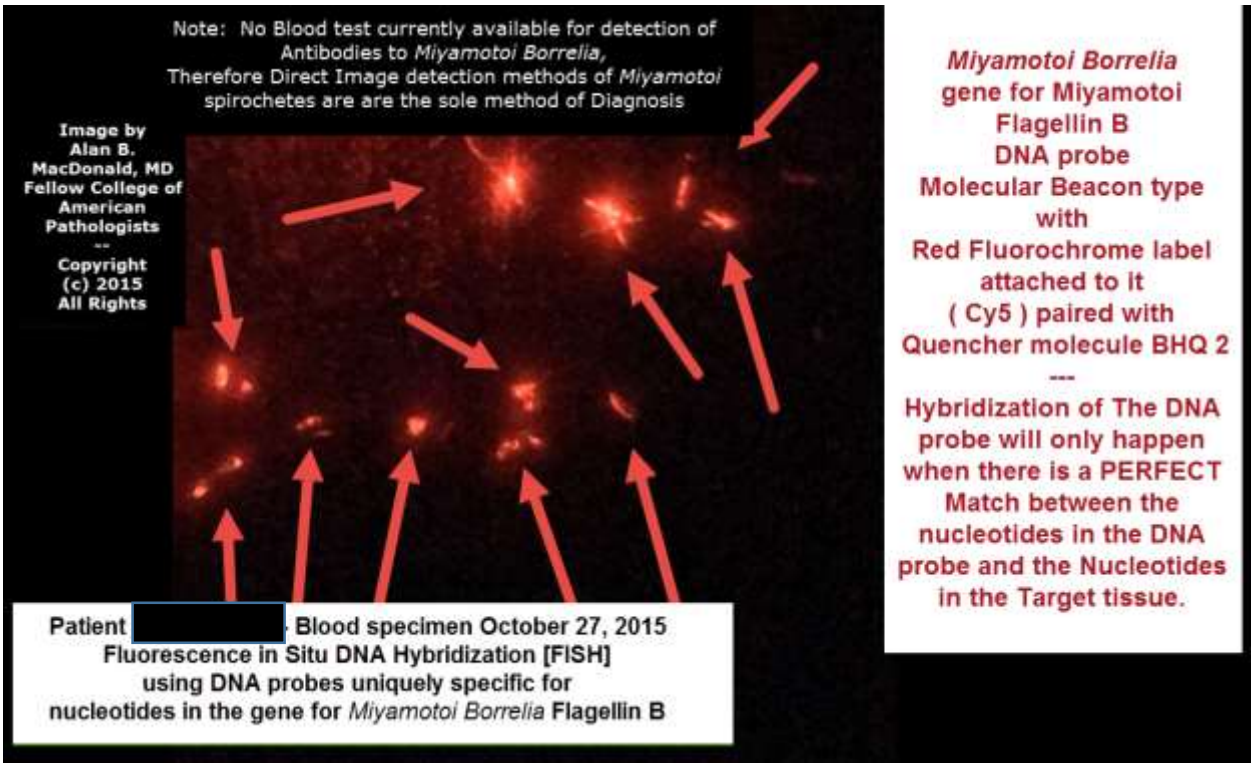
Female Adult with Chronic Lyme Borreliosis, Oslo Norway – Blood study with Borrelia specific DNA probes using
Fluorescent in Situ Dna Hybridization [FISH method] to detect spirochetes of Burgdorferi Borrelia (sl group) and Miyamotoi borrelia
Specimen obtained October 27, 2015- All Controls for FISH method are Acceptable: Respectfully submitted, Alan B. MacDonald, MD
Fellow, College of American Pathologists



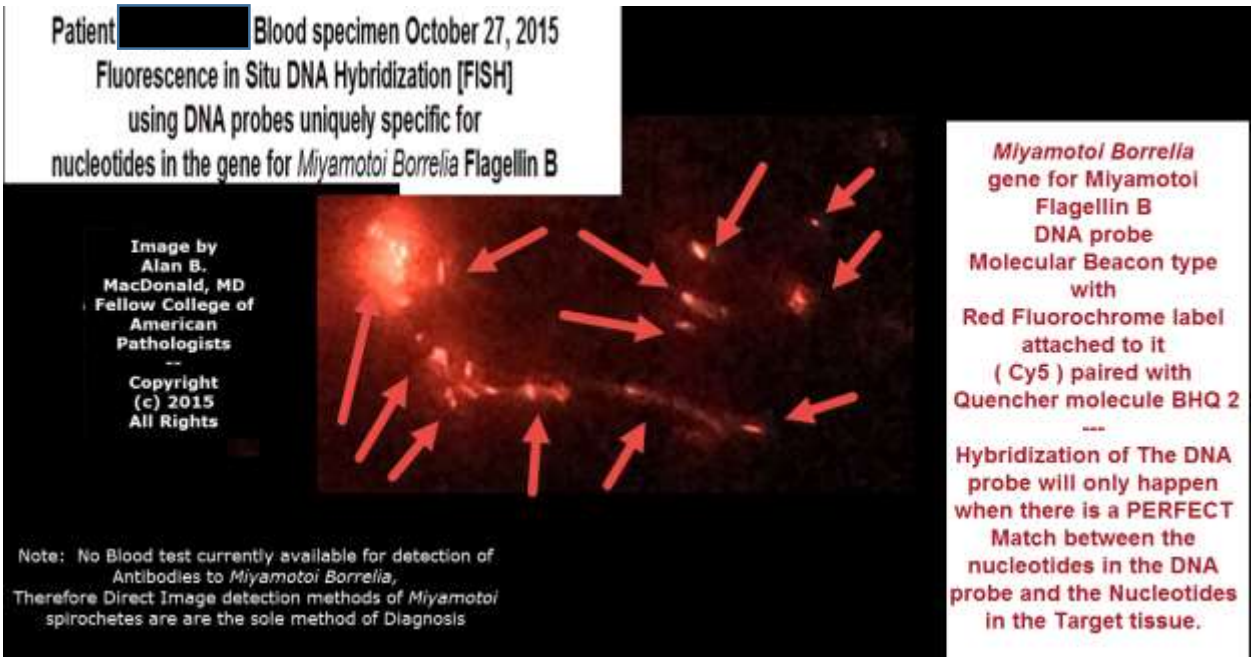
Female Adult with Chronic Lyme Borreliosis, Oslo Norway – Blood study with Borrelia specific DNA probes using
Fluorescent in Situ Dna Hybridization [FISH method] to detect spirochetes of Burgdorferi Borrelia (sl group) and Miyamotoi borrelia
Specimen obtained October 27, 2015- All Controls for FISH method are Acceptable: Respectfully submitted, Alan B. MacDonald, MD
Fellow, College of American Pathologists



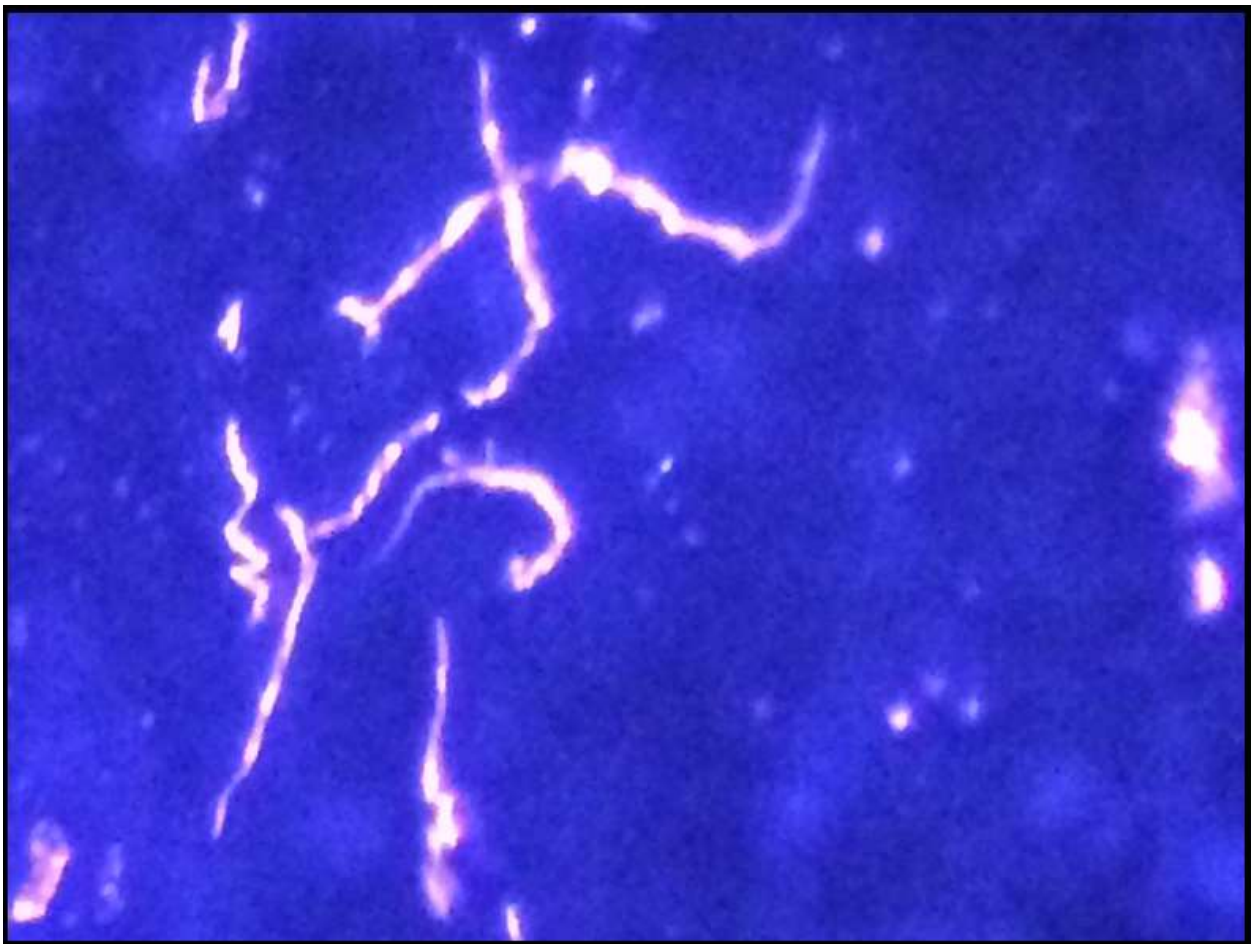
Female Adult with Chronic Lyme Borreliosis, Oslo Norway – Blood study with Borrelia specific DNA probes using
Fluorescent in Situ Dna Hybridization [FISH method] to detect spirochetes of Burgdorferi Borrelia (sl group) and Miyamotoi borrelia
Specimen obtained October 27, 2015- All Controls for FISH method are Acceptable: Respectfully submitted, Alan B. MacDonald, MD
Fellow, College of American Pathologists



Female Adult with Chronic Lyme Borreliosis, Oslo Norway – Blood study with Borrelia specific DNA probes using
Fluorescent in Situ Dna Hybridization [FISH method] to detect spirochetes of Burgdorferi Borrelia (sl group) and Miyamotoi borrelia
Specimen obtained October 27, 2015- All Controls for FISH method are Acceptable: Respectfully submitted, Alan B. MacDonald, MD
Fellow, College of American Pathologists



Female Adult with Chronic Lyme Borreliosis, Oslo Norway – Blood study with Borrelia specific DNA probes using
Fluorescent in Situ Dna Hybridization [FISH method] to detect spirochetes of Burgdorferi Borrelia (sl group) and Miyamotoi borrelia
Specimen obtained October 27, 2015- All Controls for FISH method are Acceptable: Respectfully submitted, Alan B. MacDonald, MD
Fellow, College of American Pathologists



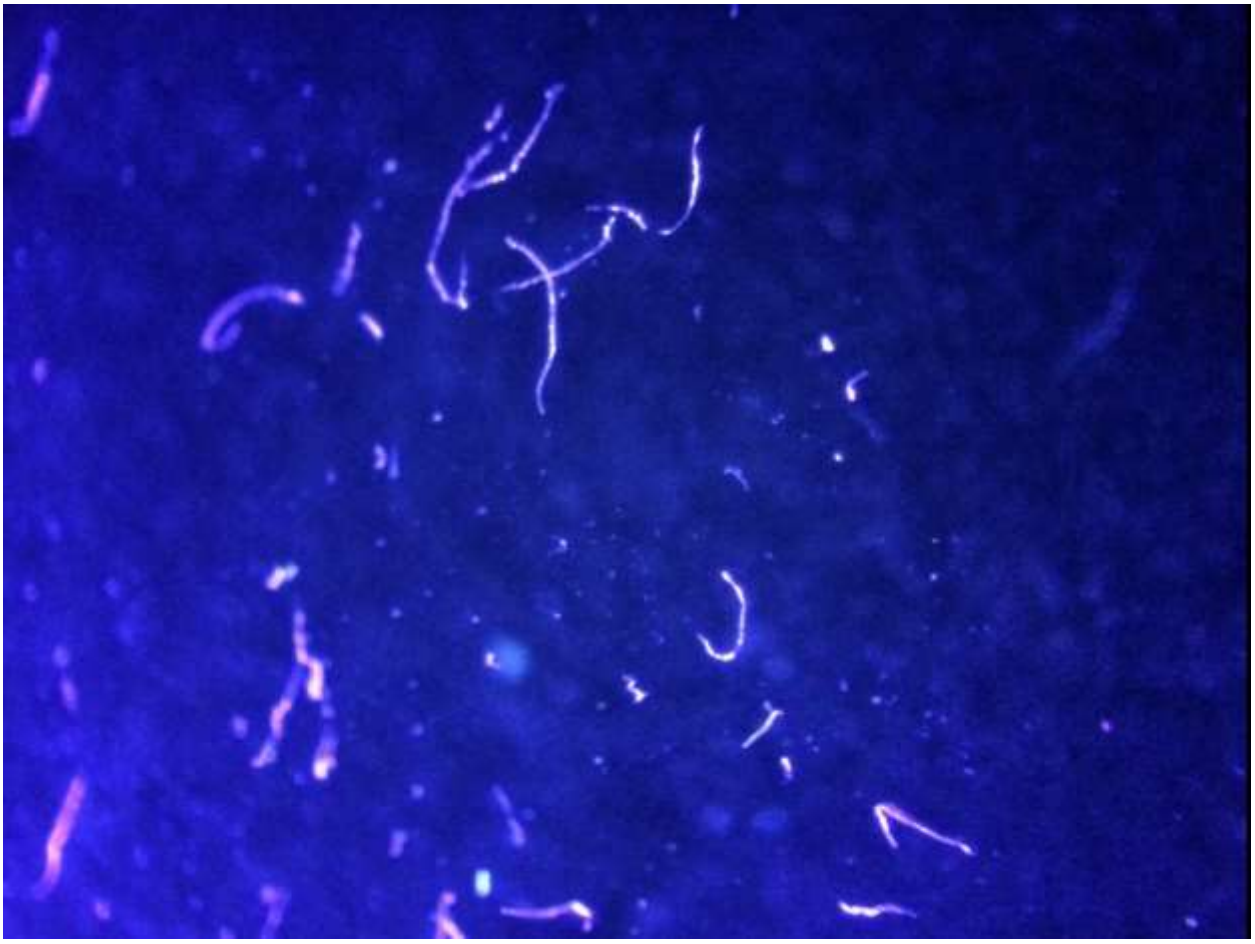
Note: Spiral microbes and dot-like granular microbes

Ethidium Bromide Staining of Peripheral Blood Smear: [EtBr stain- does not define species]

EtBr stains all double strand DNA (dsDNA)

EtBr is also utilized to stain DNA bands on Agarose gels after electrophoresis to detect

DNA amplified in the PCR reaction



Note: Spiral microbes and dot-like granular microbes

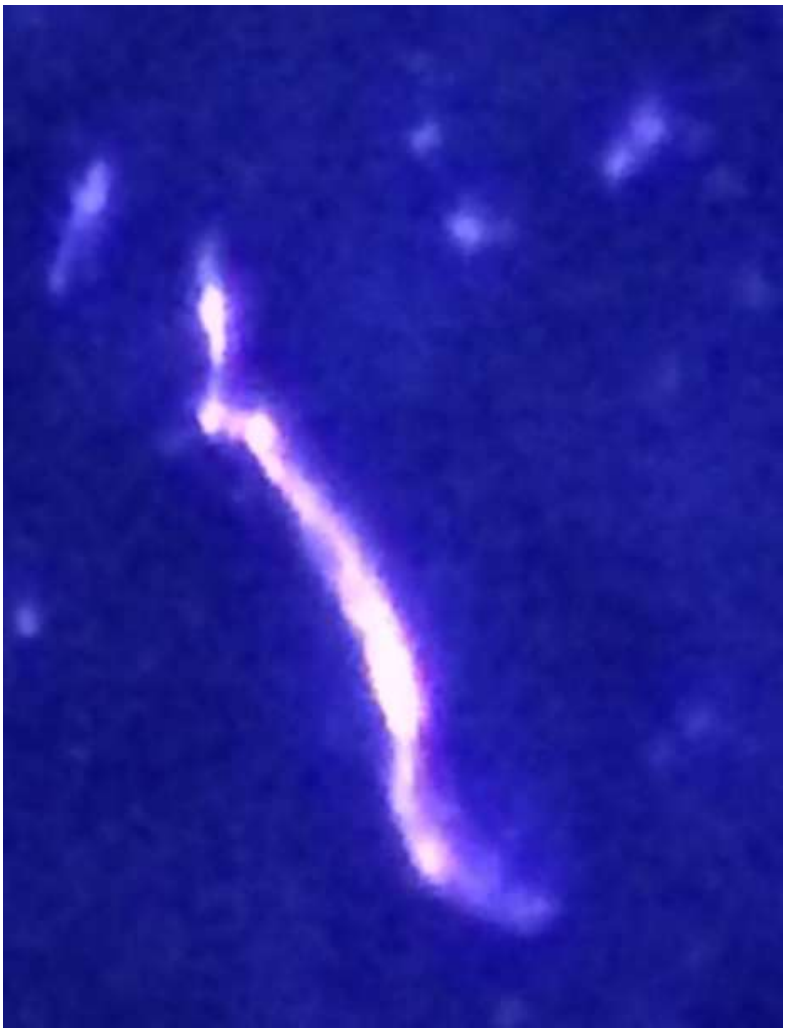
Ethidium Bromide Staining of Peripheral Blood Smear: [EtBr stain- does not define species]

EtBr stains all double strand DNA (dsDNA)

EtBr is also utilized to stain DNA bands on Agarose gels after electrophoresis to detect

DNA amplified in the PCR reaction.

Female Adult with Chronic Lyme Borreliosis, Oslo Norway – Blood study with Borrelia specific DNA probes using
Fluorescent in Situ Dna Hybridization [FISH method] to detect spirochetes of Burgdorferi Borrelia (sl group) and Miyamotoi borrelia
Specimen obtained October 27, 2015- All Controls for FISH method are Acceptable: Respectfully submitted, Alan B. MacDonald, MD
Fellow, College of American Pathologists



Note: Spiral microbe [1] and dot-like granular microbes

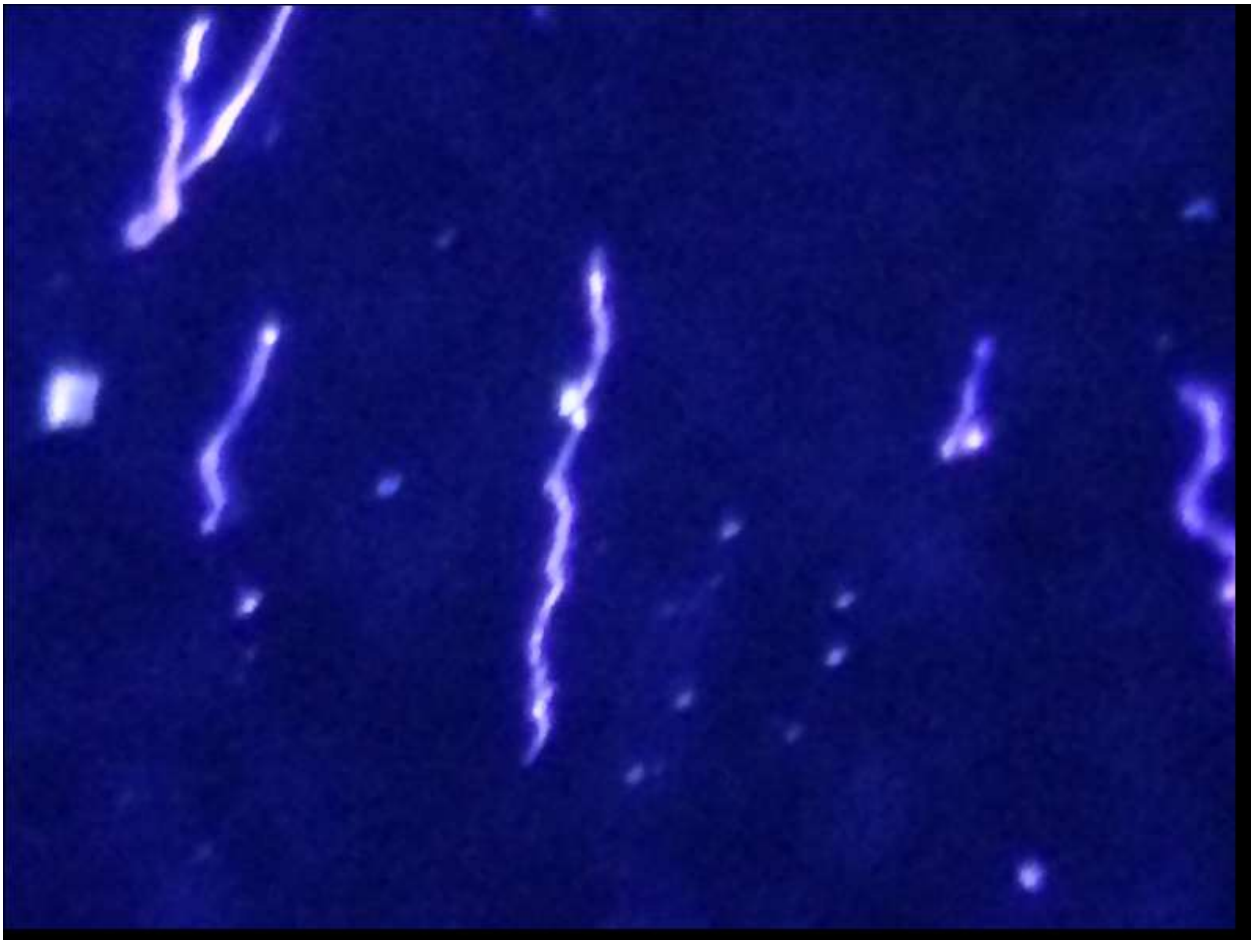
Ethidium Bromide Staining of Peripheral Blood Smear: [EtBr stain- does not define species]

EtBr stains all double strand DNA (dsDNA)

EtBr is also utilized to stain DNA bands on Agarose gels after electrophoresis to detect

DNA amplified in the PCR reaction.

Female Adult with Chronic Lyme Borreliosis, Oslo Norway – Blood study with Borrelia specific DNA probes using
Fluorescent in Situ Dna Hybridization [FISH method] to detect spirochetes of Burgdorferi Borrelia (sl group) and Miyamotoi borrelia
Specimen obtained October 27, 2015- All Controls for FISH method are Acceptable: Respectfully submitted, Alan B. MacDonald, MD
Fellow, College of American Pathologists



Note: Spiral microbes and dot-like granular microbes

Female Adult with Chronic Lyme Borreliosis, Oslo Norway – Blood study with Borrelia specific DNA probes using Fluorescent in Situ Dna Hybridization [FISH method] to detect spirochetes of Burgdorferi Borrelia (sl group) and Miyamotoi borrelia Specimen obtained October 27, 2015- All Controls for FISH method are Acceptable: Respectfully submitted, Alan B. MacDonald, MD Fellow, College of American Pathologists

Summation: DATA presented –

See References at the End of this report from the peer reviewed literature.

Experimental Methods and Controls

Display of Positive Controls for Molecular Beacon DNA probes

Miyamotoi Beacon DNA Probes [Miyam. FlaB = red]

Miyamotoi DNA probe glpQ was temporarily out of stock at

The time of this Case study.

Burgdorferi Beacon DNA probes bb0 0740gene {ORF} [FITC = green]

Ethidium Bromide {EtBr} staining of blood films as an Additional Quality control method

To independently verify that Spirochetes Containing Double Strand DNA

Are present in the blood of Siw Hansoon .

Note: EtBr intercalates between double strand DNA- of ALL life forms

No species is assigned with EtBr staining; It is used to infer the possibility of

Presence of additional microbes which are not detected by Molecular Beacons

Utilized in this research.

Research Findings:

1. Double infection: simultaneous presence of ***Miyamotoi Borrelia*** and ***Burgdorferi Borrelia***
2. Heavy load of infectomes as granular and cylindrical, spiral units

This Research is conducted at the request of [redacted], Oslo, Norway.
Blood specimens were directly obtained from the patient by Alan B. MacDonald, MD
In Naples, Florida.
Strict confidentiality of research Results is maintained

Research Study – Pro bono in the interest of the patient.

Respectfully submitted,

Alan B. MacDonald, M.D.
Fellow, College of American Pathologists [FCAP]
Report date February 1, 2016

Dear Dr. MacDonald,

Please find this letter in response to your request for my opinion with respect to your findings and autopsy report submission for PATIENT IDENTIFIER C.W.B, JR.: DATE OF BIRTH APRIL 28,

1925. After careful review of your micro pictographs and your report findings, I definitively concur with your conclusions that tissues sampled in your report contained Borrelia burgdorferi sensu lato and Borrelia miyamotoi organisms.

Methods used in the report:

As you know I am familiar with the FISH (in situ hybridization) technique and have used several of the same molecular beacons (DNA / protein targets) that you have used in this case to definitively and directly identify the various Borrelia contained and presented in the numerous case images.

It is my opinion that the FISH assay used in the production of images contained in this report is perhaps not only the most powerful and precise tool with respect to the identification of specific DNA or protein sequences of the organism of interest, but also allows for the direct visualization of the organism itself, in this case Borrelia, contained in tissue samples, and in this case further verifies the Borrelia in their in situ hallmark forms.

I know Dr. MacDonald 's probes have been validated by himself and other researchers to detect only the organisms of interest. In this case the beacon probes were specifically designed to bind and emit fluorescence only if hybridized (matched up and bound to) protein sequences of Borrelia burgdorferi sensu lato (specifically genospecies B. burgdorferi, B. afzelii and B. garinii), which are the causative agents of Lyme disease, as well as Borrelia miyamotoi, another tick borne Borrelia organism. I know the DNA/protein sequences underlying these probes to be validated not to occur in other organisms.

Further adding to the certainty of Dr. MacDonald 's findings is the fact that these particular molecular beacon probes are designed to bind only to fully complementary sequences found in the specimen. A mismatch of a single base-pair will not allow hybridization (binding) to take place and therefore fluorescence is absolutely dependent on an exact probe / sample match.

Additionally, I know that in development of beacons used in this case, proper measures were taken in order to adjust the signal-to-noise ratio to eliminate possible auto-fluorescence and background noise, thus assuring fluorescence only emanates from stable probe / target matches. While some conditions such as temperature, pH, and the type of salts used, can affect any such molecular assay, Dr. MacDonald 's use of appropriate side by side controls assures that what is demonstrated in the images of this report are, without doubt, the Borrelia of the genospecies and subspecies associated with the specific probes used.

Images:

As to the report images, they also demonstrate hallmark morphology and architecture of Borrelia found in both CSF and in brain tissue. While the report does not contain microscope objective magnifications or image processing magnifications, nevertheless it is evident that the Borrelia have appropriate size and shape of the known multiple forms of Borrelia found in vivo / ex-vivo.

Specifically, the intact spirochetal forms range from 5-30 × 0.2-0.3 µm as demonstrated in the case images. Unlike the spiral forms found in artificial tissue culture medium, some Borrelia in this case present as typical long cylinders, which are uncoiled versions, as they were exposed to various bodily fluids and tissues in vivo.

Case images further demonstrate a typical inner compartment of the spirochetes that fluoresces a central bright green core dense with DNA as tagged by the BB 0740 inner membrane target beacon. A pallid green outer compartment is also demonstrated with typical variable thickness noted due to shedding of blebs containing DNA from the outer cell membrane and occurring along the spirochete cylinder. The unique thin endoflagella of the Borrelia wind longitudinally along the spirochete cylinder and are clearly demarked by the intense green fluorescence provided by a perfect match of the BB 0147 molecular flagellar beacon with the flagella DNA.

Other morphological forms of Borrelia are clearly demonstrated in the case images as well, which are typically found in ex-vivo environment. In the brain tissue (hippocampus) sections one can see round bodies (cysts) which when living are less motile and biofilm-like colonies of Borrelia.

Some of what Dr. MacDonald describes as “small fragments of ***Borrelia burgdorferi*** Spirochetes” are in fact protein or DNA fragments specific to Borrelia burgdorferi. Some of the small rounded structures with an intense green central DNA core are actually Borrelia cysts and not solely transverse cross sections of Borrelia as produced by tissue monolayer preparation. Cross sections or transversely cut spirochetes are however also imaged and the inner cell membrane can be demonstrated by the small fluorescent rings (tagged by the inner membrane beacon BB 0740) with surrounding less intense fluorescence.

Borrelia cysts are known to originate by transverse constrictions on single spirochetes forming a string of pearls that later break in to individual cysts. The formation of these round bodies or

cysts can be seen in the higher magnification images of spirochetes contained in these case images. Several infective cyst units are produced by transverse divisions which usually starts at the ends of the spirochete, and are demonstrated particularly well in the first case image as a bright rounded bleb at one end with several bright fluorescent blebs and incongruous fluorescence seen along the length of cylindrical spirochete.

Multiple case images of both the Borrelia burgdorferi sensu lato (fluorescent green) and the B. miyamotoi (fluorescent red and yellow) demonstrate well colonies of Borrelia surrounded by an adherent polysaccharide-based matrix or biofilm. In this case, biofilm-like colonies are clearly demonstrated in the hippocampus region, an area of the brain associated with memory and with atrophy in some dementia patients. Again, as these probes are designed to fluoresce only when bound to fully complementary sequences found in the specimen and are further designed

to eliminate all possible background fluorescence, the pallid green, red and yellow fluorescence film surrounding distinct spirochetes and round body forms of Borrelia in the case images must represent as stated in this case "**Extracellular matrix material** composed in part by **Extracellular DNA** from once living but now dead ***Borrelia*** spirochetes".

Lastly, I must remind you that I am not a medical doctor and nothing contained herein either expressly stated or implied is intended or constitutes a medical diagnosis, nor implies specific cause of death. However, as a microbiologist and after careful review of the FISH molecular procedure used to derive images contained in the autopsy report and after careful review of all case images, it is my expert opinion that tissues sampled and presented in your report, with upmost certainty, did in fact contain species Borrelia burgdorferi sensu lato and Borrelia miyamotoi organisms in their various morphological forms. I can ratify the validity of the images which appear in Dr. MacDonald's report and concur that Dr. MacDonald's Molecular Beacons used for FISH method imaging of Borrelia in diseased tissues in this case are absolutely specific to their respective targets and are in my opinion the gold standard for identifying Borrelia in ex- vivo tissue.

Submitted Respectfully,

Dr. Jennifer Souders, D.PT

References:

Fluorescence in Situ DNA Probe Hybridization Method to detect Borrelia DNA

1. Hammer B, Moter A, Kahl O, Alberti G, Göbel UB (2001)
Visualization of ***Borrelia burgdorferi*** sensu lato by fluorescence in situ hybridization **(FISH)** on whole-body sections of ***Ixodes ricinus*** ticks and gerbil skin biopsies. Microbiology 147 (Pt 6):1425–1436, PMID 11390674
2. MacDonald, A. B. "***Borrelia burgdorferi*** tissue morphologies and imaging methodologies." *European journal of clinical microbiology & infectious diseases* 32.8 (2013): 1077-1082.
DOI: 10.1007/S10096-013-1853-5

This Research is conducted at the request of the Patient:
[redacted], Oslo, Norway, who has been diagnosed by USA Expert Medical Consultants; [Diagnosis= Chronic Borreliosis,] and who has been prescribed Antibiotic Combination Therapy which is absolutely required to Treat Chronic Borreliosis. Siw Hansson is currently under the care of Richard Horowitz, MD, an Internationally recognized expert in the diagnosis and patient treatment of Tick Borne Illnesses.

Image based evidence to establish the persistence of Borrelia species (mixed Miyamotoi borrelia and burgdorferi borrelia in the blood of [redacted] of Oslo, Norway,
Is absolute pathological evidence of the continued presence of living Borrelia spirochetes in her blood.

Research Study – Pro bono in the interest of the patient and the family.
Molecular Interrogation of Formalin Fixed Paraffin Embedded Autopsy Tissue with FISH method using Molecular Beacon Type DNA probes specific for ***Borrelia*** Species as identified above.
Respectfully submitted,
Alan B. MacDonald, M.D., Fellow, College of American Pathologists [FCAP] {FASCP}
Board Certified in Anatomic Pathology and in Clinical Pathology –
American Board of Pathology, USA.

Female Adult with Chronic Lyme Borreliosis, Oslo Norway – Blood study with Borrelia specific DNA probes using
Fluorescent in Situ Dna Hybridization [FISH method] to detect spirochetes of Burgdorferi Borrelia (sl group) and Miyamotoi borrelia
Specimen obtained October 27, 2015- All Controls for FISH method are Acceptable: Respectfully submitted, Alan B. MacDonald, MD
Fellow, College of American Pathologists