FIRST APPLICATION OF AN IMPROVED-RESOLUTION DARK-FIELD MICROSCOPY SETUP, COMBINED WITH A NOVEL DIRECT-STAINING LIVE IMMUNOFLUORESCENCE INVESTIGATION METHOD, VISUALISING THE MOTION OF *BORRELIA BURGDORFERI* SPIROCHETES

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ABSTRACT
Dark-field microscopy has been increasingly applied in diagnostics in recent years, for example in Lyme borreliosis, due to the growing concerns about the diagnostic utility of standard serological tests. Although the diagnostic value of microscopic methods is still debated due to the lack of proper clinical testing and the definition of the diagnostic cutoff levels, it is important that the spirochetes causing the disease can be visualized from body fluids via dark-field investigation. Additionally, a specific identification of *Borrelia* is still not routinely possible, visual observation is usually fortified with additional PCR investigations. We are publishing the pictures from the first-time application of an enhanced dark-field illumination technique that improves the resolution and picture quality of digital recordings. The same illumination setup, combined with a novel live staining of *Borrelia burgdorferi* with cleaned, FITC-labelled anti-Borrelia antibodies, has produced remarkable recordings of the same spirochetes, in natural motion. All experiments were performed on remnant samples of DualDur kit laboratory investigations.

Keywords: Dark-field microscopy; immunofluorescence; *Borrelia burgdorferi*; venous blood; DualDur kit.

Primera aplicación de una configuración de microscopía de campo oscuro de resolución mejorada, combinada con un novedoso método de investigación de inmunofluorescencia viva de tinción directa, visualizando el movimiento de las espiroquetas de *Borrelia burgdorferi*.

RESUMEN
La microscopía de campo oscuro se ha aplicado cada vez más en el diagnóstico en los últimos años, por ejemplo, en la borreliosis de Lyme, debido a la creciente preocupación por la utilidad diagnóstica de las pruebas serológicas estándar. Aunque el valor diagnóstico de los métodos microscópicos todavía se debate en la falta de pruebas clínicas adecuadas y la definición de los niveles de corte diagnóstico, es importante que las espiroquetas que causan la enfermedad se puedan visualizar a partir de fluidos corporales a través de la investigación de campo oscuro. Además, una identificación específica de *Borrelia* aún no es posible de forma rutinaria, la observación visual suele fortalecerse con investigaciones adicionales de PCR. Estamos publicando las imágenes de la primera aplicación de una técnica mejorada de iluminación de campo oscuro que mejora la resolución y la calidad de imagen de las grabaciones digitales. La misma configuración de iluminación, combinada con una novedosa tinción en vivo de *Borrelia burgdorferi* con anticuerpos anti-Borrelia limpios y marcados con FITC, ha producido grabaciones notables de las mismas espiroquetas, en movimiento natural. Todos los experimentos se realizaron en muestras restantes de las investigaciones de laboratorio del kit DualDur.

Palabras claves: Microscopía de campo oscuro; inmunofluorescencia; *Borrelia burgdorferi*; sangre venosa; Kit DualDur.

INTRODUCTION

Due to their special shape and movement, as well as their larger-than-average length among bacteria, spirochetes were among the first to be identified by microscopy (*Treponema* species, *Borrelia anserina*).

The Dutch merchant Antonie van Leeuwenhoek observed spirochetes as early as 1683 by microscopic examination. By the middle of the 19th century, dark-field microscopy became the most frequently used method for the detection of spirochetes.

In 1868, Otto Obermeier identified a spirochete, *Borrelia recurrentis*, as the causative agent of recurrent fever, making it the first human disease associated with a microbe. The causative agent was detected from a blood sample by microscopy [1].

The name *Borrelia* was given in the twentieth century in honor of Amédée Borrel (1867–1936), who first documented the difference between borreliae, namely *B. anserina*, and another known species of spirochetes belonging to a different family, *Treponema pallidum* [1].

Robert Koch also demonstrated his famous postulates with the help of spirochetes (relapsing fever borreliae) [2].

Dark-field microscopy techniques have been improved, and since 1909, dark-field examination applied on unstained, unfixed preparations is the standard method to diagnose the infection caused by *Treponema pallidum* spirochetes (Syphilis) [3].

After unsuccessful studies using the then most modern means of laboratory examinations, Willy Burgdorfer was able to detect the causative agent of Lyme borreliosis by simple dark-field microscopic examination in the hitherto unknown spirochete found in ticks collected in the Long Island area, and in the samples of patients, and published the results in 1982 [4].

From the 1990s novel visualization techniques (e.g. electron- and confocal microscopy), serology, and polymerase chain reaction (PCR) were considered the new promising methods replacing dark-field investigation in scientific and laboratory practice. Nevertheless, the high percentage of seronegative [5] cases of Lyme borreliosis gave rise to the rediscovery of dark-field microscopy in both diagnosis and scientific research, at times combined with PCR [6] [7].

However, it was needed that a standardized technique be introduced that is easily carried out at the everyday laboratory setting.

Pseudo-spirochetes, created from human cell remains cause a problem of distinction [8] [9], while conventional immunofluorescence is elaborate and there is a high chance that spirochetes are washed away from the glass surface or damaged otherwise during the process [10].

The methods and devices outlined in this research article help avoid the above problems and contribute pictures with improved visual clarity, and show the natural behavior of spirochetes, also with a proof of specificity.

MATERIALS AND METHODS

Specimens

All samples were collected as remaining samples from DualDur laboratory investigations, courtesy of Lyme Diagnostics Ltd. Hungary, Europe. 4ml of human venous blood was taken onto 4ml of DualDur cell technology medium, mixed, and stored at 4-8 °C, transported for a maximum of 24 hours at room temperature, prepared and investigated within 172 hours of phlebotomy.

Samples were taken from subjects who were suspected of having a *Borrelia* infection or other type of coinfections like *Bartonella* or *Babesia*.

DualDur cell technology medium contains among others selective nutrients for *Borrelia burgdorferi*, glucose, human cell medium RPMI 1640, anticoagulant EGTA (ethylene glycol tetraacetic acid), agents to stiffen the membrane of blood cells but not that of bacteria (e.g. caffein salts), thus avoids decomposition of blood cells and creation of pseudo-spirochetes.

Preparation method: Borrelia concentration

Borrelia content of the samples was extracted using the DualDur kit and preparation method (Composition of the liquid and the preparation outlined in detail in the Hungarian priority Patent P1900099, PCT WO2020194003) [11] The low-speed centrifugation (3 300 g) separates the blood cells from the sample, 4.5 ml of the supernatant is subjected to high-speed centrifugation (15 500 g), After discarding the supernatant, 2.5 µl of the resuspended sediment is examined under the dark-field microscopy setting described below.

Preparation method: Immunofluorescent staining

Version 1: GeneTex Borrelia burgdorferi antibody FITC reagent (GeneTex, Inc., North America), that contains cleaned antibodies conjugated with fluorescein isothiocyanate (FITC) was used for the experiment. The specificity of the reagent was previously tested in a separate experiment using 3 different *Borrelia* strains as positive and one *Treponema* strain as negative control (DSMZ, Germany), as only binding to *B. burgdorferi sensu stricto*, *B. garinii* and *B. afzelii*. The GeneTex reagent, original concentration 4-5 mg/ml, was further dissolved 1:40 in PBS and stored at 4-8°C, vortexed right before use for 2 minutes at 3500 rpm. Borrelia-containing sediment of the DualDur kit sample (prepared according to the Preparation method: *Borrelia* concentration) is mixed in a dark room 1:2 with the diluted GeneTex reagent solution, vortexed at 3500 rpm for 2x1 minutes, then incubated at 4-8°C for 1-4 days. Right before the investigation, the sample is vortexed for one minute at 3500 rpm and 2.5 µl of the sample is investigated.

Version 2: The above diluted GeneTex reagent solution is applied in a different way. 2.5 µl of *Borrelia*-containing sediment of the DualDur kit sample (prepared according to the Preparation method: *Borrelia*-concentration) is placed on a slide and covered by an 18x18mm cover slide. The sample is partly dried out at 30-35°C for 15 minutes in a dry environment (<40% relative humidity, RH) so that about 50% of the area looks transparent (liquid), and the other 50% looks dried out. 4 µl of the diluted GeneTex reagent solution is gently applied to the edge of the cover slide from one side, so it is spread under the cover by capillarity. The sample is incubated for 1-2 days at 4-8°C, at >90% RH, and investigated immediately after removing from the fridge.

Devices

The specific device used is described in more detail in the Hungarian priority patent P2000014 (WO2021144596) [12].

Nikon Ni-U microscope with a darkfield condenser, 60x objective, originally equipped with a halogen light source was modified. Illumination with 4W Cree light emitting diode (LED, 450 nm wavelength) was applied with a corresponding driver and collimation solutions (own development). LED power was adjusted between 1200-1800 mA. Important note: a conventional epi-fluorescent microscope setup and illumination could be used for evaluating the samples; however, this will only detect the borreliae that show the highest levels of reaction with the FITC-conjugated antibodies, also fading more rapidly due to a higher level of illumination power being necessary.

For black&white pictures two cameras were used from Basler AG, Germany, with small pixel sizes (2.2 to 3.8 um), Basler MED acA3088-57um (without further magnification) and acA4112-30um optionally with a further 4x magnification insert. Own-developed DualDur Diag Recorder software was used with exposition times 0.1-0.3 seconds, 5-18 gain.

Color pictures were taken with the Basler acA4112-30ucMED camera. A conventional green optical filter was placed in the phototube before the camera. Recording of the pictures of this camera was done with Basler Pylon viewer 6.0-6.3 software versions (Basler AG, Germany), exposition time 0.4 seconds, gain 0.7-2. The microscope was equipped with an epi-fluorescence FITC kit (FITC/Alexa Fluor 488/Fluo3/Oregon Green), which was

used in some photos to completely exclude the blue excitation light, while not removing the conventional green filter either (triple filtering). Top illumination supplied with the epi-fluorescence kit was not applied.

Investigation

2.5 µl of the sample was investigated in each case, starting from the top left corner of the cover slide and going in an orderly manner towards the right, and then down and back left in a snake-like pattern. Steps of 3-5 mm (looking at the display of the digital stage) were taken between two adjacent rows to avoid investigating the same objects again – liquid currents and moving objects were expected. Objects were photographed and selected. No other editing was done except for cutting.

Ethics

The study was conducted in accordance with the Declaration of Helsinki. The investigations were carried out on the deidentified remnants of human specimens collected for routine clinical analysis that would otherwise have been discarded. Such investigations are considered under the Hungarian law "non-interventional' studies, using a CE-marked device. No requirement for ethical approval is stated according to the EU In Vitro Diagnostics Regulation (IVDR, Regulation EU 2017/745), as this is not a post market investigation or a performance evaluation.

RESULTS AND DISCUSSION

Borrelia burgdorferi detection by dark-field illumination

There is a wide-ranging discussion about whether or not *Borrelia* is present in body fluids throughout the whole course of Lyme disease. It is certainly an advantage for the blood-borne pathogens to be present in or re-enter the blood so that the vectors, feeding on blood, can be infected and ensure the spreading and survival of the bacteria. Completely excluding the presence of the pathogen in the blood would render infection of the tick vector impossible. Borrelial infections cause surges of bacteria across the body in humans, during which the density varies between 10^7, even 10^9 and a few thousand bacteria/ml in different organs and the blood. In relapsing-fever type *Borrelia* (e.g. *B. recurrentis*), the surge comes in 10-14 days and is concurrent with up to 10^7 bacteria/ml observed in serum, and a marked worsening of the symptoms, usually including fever [13]. No wonder that this type of spirochete was firstly identified due to its large concentration in blood, so no extra concentration step was necessary to discover them by microscopy. This concentration cited by literature means that there are 20-50 bacteria in every field of view of a conventional microscope slide (2-5 µl investigated under a cover slide hosting 1000 fields of view), though most of them being covered by blood cells, while the plausible limit of detection is one bacterium in 50-100 fields of view. Hence, the discovery of bacteria can be expected.

Borrelia burgdorferi sensu lato, on the other hand, produces surges of less than 10^6 bacteria, which means that without further concentration, even the highest level can be below the limit of detection [14]. At the same time, there may be very mild symptoms [15], their waving might be unnoticed, or the infection may go asymptomatic [16] for years.

The original hypothesis behind using the DualDur concentration method for our experiment was the hope that the scarce *Borrelia burgdorferi* would be visible in a large proportion of blood samples from symptomatic patients. This hypothesis was set because the DualDur kit uses a concentration step of up to 100-1000x, removing the blood cells from 4ml of body fluid and concentrating the bacterial content of the plasma into 4-40 µl of liquid.

Importantly, the detection of a few or many borreliae in a patient sample has no diagnostic value without a validated cutoff level to define positive or negative test values. Hence our experiment clearly focused on recording the

motion of objects in the samples and proving that they are most probably *Borrelia burgdorferi* by immunofluorescence, merging the two methods, dark-field and fluorescence microscopy, in one examination.

In a preliminary experiment (publication in progress) the authors validated that the concentration of the bacteria (after removing the blood cells) from 4ml blood sample into 30 µl of sediment will set the limit of detection of microscopy to 2x10^3 bacteria/ml of the original sample.

In any case, the used methods should avoid forming pseudo-spirochetes and be able to detect moving spirochetes as opposed to stationary objects or objects moved by the liquid currents and Brownian motion [9]. It has been noted that such objects create a false impression of a *Borrelia* infection and raise debates about the utility of microscopy in diagnostics in general [17].

A further improvement that made the pictures possible was the better resolution of the microscope. Previously, with white-light halogenic illumination, the angular resolution of the darkfield microscope was around the width of *Borrelia* (~400 nm). This means that even with the best cameras, only the thickest examples of *Borrelia* were seen. The discovery outlined in the patent P2000014 detected that the critical behavior of Abbe's equation in case of a dark-field illumination works in the following two directions: (i) the reduction of the illuminating wavelength improves the resolution, whereas (ii) the increasing of numerical aperture (NA) is limited to about 0.85-0.95, because of the direct light entering the objective at higher NA. Dark-field microscopy is based on diffraction, the so-called Tyndall effect, where the direct illuminating light passes through the sample in a cone-shape and avoids the objective, so only the low-intensity light scattered by the double-refractory biological objects is seen. Objectives with a higher NA collect the light from a larger angle, hence even the direct light is collected, which makes the background grey and the background suppresses the scattered light of importance. At the same time, the depth of field is reduced by decreasing the wavelength, so it cannot be lowered beyond any limit. The sweet spot is the technical setting described in the patent, around 450 nm wavelength and 0.85-0.95 NA. Since dark-field microscopy is based on diffraction, the white illuminating light passing through a sample object is split into separate beams by the wavelength, thus adding a rainbow-colored edge to every object. This forms a halo in the black&white pictures, which can be avoided by a monochromatic light source. Thus, the monochromacy of the light source eliminated the halo effect usually caused by the diffraction of white light at the edge of objects.

Improved resolution of the camera picture, sharper images and higher sensitivity of the chip (quantum efficiency of >0.8), pixel sizes below 4 microns achievable with Basler B&W cameras, enabled us to record the natural motion and microstructure of borreliae in real time. Only after the first investigations did we discover that a large number of objects had not been seen before due to their width being below the angular resolution of the traditional dark-field microscope and due to the lower sensitivity of the camera. It goes beyond the scope of this article, but it should be mentioned that the width of *Borrelia* is probably not just an anatomic feature, but also refers to their biological activity: *Borrelia* longer present in the host tend to cover themselves with the unit membrane of the host [18] or by polysaccharides [19], but the young and multiplying individuals may lack these features. Hence the width may correlate with the biological activity.

Figures 1 and 2 cover the findings, in which the researchers deliberately selected only those spirochetoid objects that clearly demonstrate an autonomous motion similar to that expected from spirochetes.

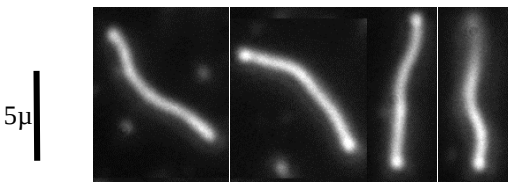


Figure 1. Medium-sized spirochetoid object with active motion identified as *Borrelia burgdorferi sensu lato*

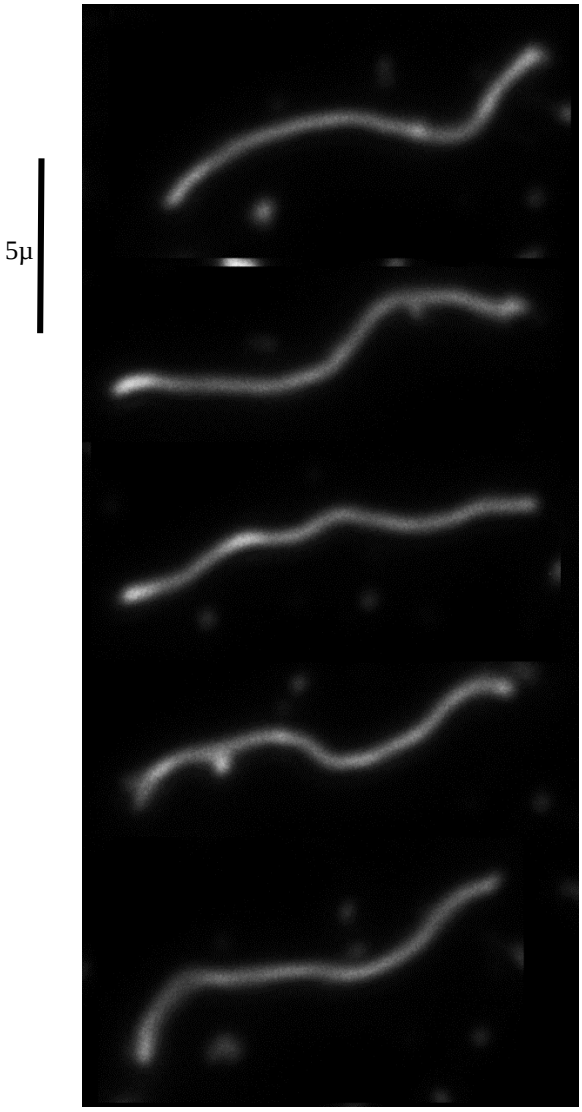
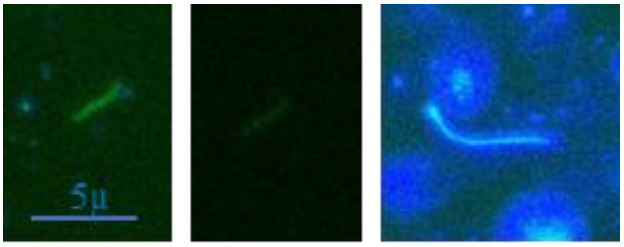


Figure 2. Long spirochetoid form with active motion, identified as *Borrelia burgdorferi sensu lato*
Borrelia burgdorferi detection in the sample treated with FITC-labelled anti-Borrelia antibodies

Pictures were recorded predominantly after preparing the sample with Version 1. As confirmed by adding the epi-fluorescent set (filters and dichroic mirror), the moving cyan and greenish-blue objects contain a green note and therefore indicate the binding of the antibodies in a small quantity. Figure 3a shows the same object with a single conventional green filter and 3b with the same green filter and the epi-fluorescent mechanism added, an additional picture 3c was taken from another object without any filter, so we can see the blue-color "dark-field" picture superposed on the fluorescent dye. Reduced light intensity in 3b is due to the triple filtering, because the current solution does not yet allow to easily remove the conventional green filter.



3a **3b** **3c**

Figure 3. Immunofluorescence pictures of *Borrelia burgdorferi sensu lato* from a sample prepared with Version 1; 3a: single green filter; 3b: same object triple filter; 3c: different object without any filter.

Further pictures with the same illumination and the same conventional green filter are shown in Figure 4. Motion of the borreliae is represented by a series of still pictures. General observation is that the binding of antibodies is:

- regulated by the relative quantity of *Borrelia* in the sample, so more intensive binding occurs with less *Borrelia* being present
- increased with the better matching of the antibodies to the antigens in the sample: in some samples many unstained *Borrelia* are recorded, with very faint intensity still let through due to the wider wavelength profile of the conventional green filter.
- increases with time to a certain limit: at the concentration applied, a minimum of 24 hours is needed

See the various levels of staining in Figure 5, resulting from the superposition of a low-intensity light from the blue illumination and a varied thickness of antibody binding marked with green FITC fluorescence.

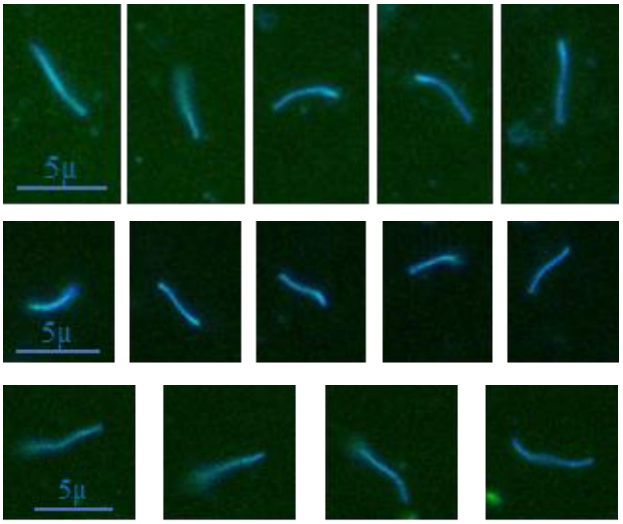


Figure 4. Immunofluorescence pictures of *Borrelia burgdorferi sensu lato* showing active motion, from samples prepared with Version 1

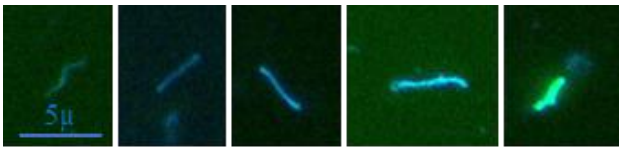


Figure 5. Immunofluorescence pictures of *Borrelia burgdorferi sensu lato* showing various levels of staining from blue to cyan and FITC green, from samples prepared with Version 1

The theory for the process of binding in the sample is as follows. Low temperature reduces the conductivity of the DualDur cell technology medium. This facilitates the binding of the antibodies. If there is a perfect match between the cocktail of antibodies and the available antigens in the sample then binding occurs fast and demobilizes the borreliae. Further binding covers the whole surface of the borreliae, and this results into an almost fully green, non-motile structure in the shape of an L, S or C. The edges are blurred (no sharp edge on either side, as opposed to pieces broken off the artificial coagulations of the antibodies) and the color is slightly less green, more with a blueish note than the artificial coagulation of the dye itself that may cause artifacts.

If the binding is less perfect or slower, then it occurs in a way that does not immobilize the borreliae. The partly successful binding results in a cyan color, bearing a greenish note, probably composed of the blue color of the dark-field illumination and the green color of the FITC. The binding sometimes only covers parts of the Borrelia. If the sample is incubated longer than 4-5 days then the antibodies may create larger antibody-antigen complexes (blebs), small bumps on the Borrelia that are then sometimes cast off the surface of the spirochete [20]. This reduces the overall intensity of the fluorescence.

Light intensity induces fading. With traditional immunofluorescence preparation, it is inevitable that the epi-fluorescence filters are applied to increase specificity because objects are not in motion. It is easier to mistake an artifact for a marked Borrelia. In this case, the investigation is carried out with a double light intensity compared to our novel method (3500 mA vs. 1500 mA) by browsing through the sample with the ocular for faster detection, and when an object is detected then the light is diverted into the camera, and pictures should be taken within 10-20 seconds before the object fades.

With our new method, there is no need to subtract the illumination wavelength, because it is sourced from below the sample by the dark-field condenser, therefore no need to apply a dichroic mirror, because there is no upper illumination. (Epi-fluorescence contains upper illumination and upper detection, this new method uses bottom illumination and upper detection.) This is why the new method can take pictures technically for more than a minute in the same position before the fading of the objects is noticed. When triple filtering is applied, then the light intensity is reduced by the filters, but still a motion picture can be recorded in some cases with the color camera due to the high sensitivity of the device, if the antibody binding is optimal in density.

Stationary pictures proving the immune response to the infection

Figure 6 shows a preparation done with Version 2. White blood cells obviously degraded some *Borrelia* by phagocytosis. Due to the preparation method, the cell membrane was burst, releasing the cell content into the plasma. The flow of the diluted reagent has taken the contents of the cells to the right. At the same time, the free-flowing antigens released due to the breaking up of the spirochete still within the cell, allowed the antibodies to be bound more thoroughly. This causes the greenish spots to the right of the burst blood cell.

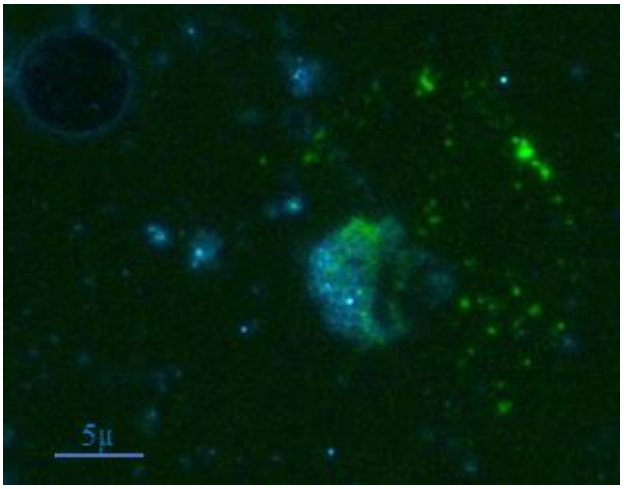


Figure 6. Proteins of degraded *Borrelia burgdorferi sensu lato* are released from a burst white blood cell and marked with the immunofluorescence reagent, a sample prepared with Version 2. The shadow of a red blood cell is seen in the top left corner.

CONCLUSIONS

Moving *Borrelia burgdorferi* spirochetes concentrated from blood samples of patients by the DualDur kit can be observed by dark field microscopy and confirmed by immunofluorescence.

Similar forms of *Borrelia burgdorferi sensu lato* have been recorded from real patient samples in various publications [6]. It has been demonstrated that the shape of borreliae is changeable according to the surrounding media and the position of flagella [21], which shape is not always the traditionally seen corkscrew shape.

Camera picture resolution from dark field microscopy can be enhanced by applying blue monochromatic illumination light, which at the same time can be used to excite FITC dyes for immunofluorescence.

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CONFLICTS OF INTEREST

Both authors are founders of Lyme Diagnostics Ltd. which is the owner of the two patents used in the investigations.

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