Application of dark-field microscopy for detection of DNA and protein

Qiuyi Tang *

School of Health Science and Engineering, University of Shanghai for Science and Technology, Shanghai, 200093, China

* Corresponding author. Email: 1719640509@st.usst.edu.cn

Abstract. Dark-field microscopy (DFM) can be used to observe living cells and microorganisms. In contrast to a bright-field microscopy, the entire field of view in DFM is dark by default and the dark background provides a high contrast. And when the researchers placed the target samples in the path of the light cone, it appeared bright because it was on an almost black background, so it shows outstanding detail. DFM is a technique to improve the contrast of specimens with poor imaging quality under conventional illumination. After the direct light is blocked by the opaque aperture in the condenser, the light passing through the sample at an inclined angle is reflected into the microscopy objective lens through diffraction, refraction and reflection, producing a bright sample image. The DFM is preferred for opaque, low transparency and low contrast specimens, especially when staining is not a viable option. DFM has the unique advantages of being easy to modify and effective, so this technology shows a diverse of different applications. Herein, this research outlines the characteristics of DFM and analyzes its application in DNA and protein detection.

Keywords: dark-field, DNA, protein, sensitivity, application.

1. Introduction

Dark-field microscopy (DFM) is effective in detecting macromolecules. DFM is a technique used to observe living, unstained cells and microorganisms with excellent results. When using bright-field microscopy, the researchers should ensure that the sample is thin, semi-transparent and the dyeing results have high contrast. But that's not enough, because if the dyeing technique is not appropriate, and the dye will kill the living cells, which will invalidate observing the behaviour and catalysis of live samples, and cause the experiment lose its value and significance. In order to improve the above limitations, the researchers can use DFM to observe the macromolecules.

Compared to the bright-field microscopy, the sample in DFM is illuminated by a light cone, which is controlled by condenser, hollow and focusing. The objective is outside of this bright area, and the light travels around the objective without actually entering the cone. Thus, by default, the entire field of view is dark, and when the researcher places the target sample in the light cone path, it appears bright because it is on an almost black background, so that it presents outstanding detail. DFM is a preferable choice for opaque, low-transparency, and low-contrast specimens.

The DFM has many useful applications in biology, metallurgical science and industry [1]. For example, it can also be used to observe the blood cells, tissue sections, bacteria and algae. The DFM is crucial, especially for low contrast samples. And also, it can be applied to characterize nanomaterials. Though DFM, the details of microorganism become clearly visible. This technology can amplify the physical and cellular structure of microorganism several times. In this way, the researchers can also see the movement of living samples, for instance the movement of bacteria and organisms in water. Besides, DFM can check the quality of different kinds of stones and metals easily, as it can stand out the faults, cracks and fractures conveniently.

The greatest advantage of DFM is that the technique is fundamentally simple, but very effective. All kinds of microscopes can be easily modified. That is to say, the microscopy still works when the desired component is embedded, especially with the addition of a patch or shield disc. It should be noted that the DFM require intense light in any case, so mirror microscopies will not be suitable. It can provide high-resolution images, which is particularly beneficial for living cells sample. With this

method, details on smooth surfaces can be highlighted well and the refraction of the sample will be minimized. Using the DFM to view samples, which normally obscured by shadows under bright-field microscopies also shows good imaging results. The DFM also have some drawbacks. For example, it is necessary to quickly test moist specimens containing living organisms, as watching for moving bacteria is crucial for testing. In addition to the sample, dust is disturbing due to the apparent scattered light. And the collected scattering intensity must still be high enough to obtain a high-quality image. To date, some probes have high imaging accuracy and have established excellent scattering efficiency, such as precious metal nanoparticles and ordered combinations of organic molecules.

Macromolecules are biological substances, which relative molecular mass is more than 5000, even more than one million. Macromolecules including DNA, proteins and polysaccharides are closely related to life activities, so the detection of macromolecules is of great practical significance. In this paper, the research background, current technology status, application field, application examples and development trend of DFM will be described.

2. applications of DFM for detection of DNA and proteins

2.1. DNA detection

Brinkers et al. constructed a new method to measure the dsDNA length in controlled environment. In this way, because dsDNA is not affected by external forces and the molecule is not confined to a two-dimensional (2D) surface, a more natural three-dimensional (3D) conformation can be used. As shown in Figure 1, the tethered particle motion (TPM) was used. The prepared particle-molecule system can show restricted Brownian motion [2]. Different mechanical properties and environmental conditions of captive molecules lead to different trajectories and states of motion. By tracking the motion of the particles, the researchers can deduce the tether properties. In their work, the researchers used gold particles (80 nanometres in diameter), where the particle size is small enough to avoid affecting the motion. These highly scattered gold nanoparticles were observed with the used DFM. The system is simple in structure and can produce images with high signal-to-noise ratio. By comparing the position distribution of the nanoparticles statistically with the results of a computer simulation experiment in which dsDNA was modelled using a wormlike chain model, the duration of the binding of dsDNA could be determined. Based on the FJC and WLC models, for long molecules, the position distribution of DNA should be Gaussian distribution, so researchers used Gaussian distribution to fit the distribution. In the TPM experiment, the researchers projected the position of particles attached to the tether in two-dimensions. The fitting results show that the simulated distribution of the experiment follows the Gaussian distribution. If the particle-substrate interaction causes significant volume repulsion, the distribution will not be Gaussian.

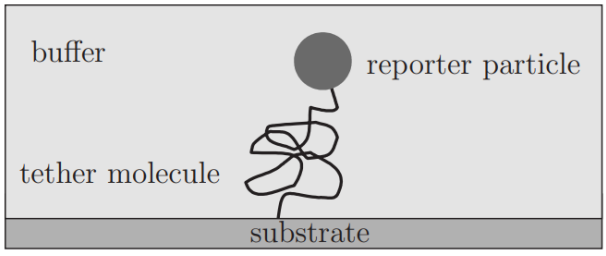


Figure 1. The principle of tethered particle motion [2].

The use of the DFM allows for smaller particle because the scattering of these particles is very noticeable against a dark background. The main feature of this method is that 45 separate, independent measurements are made: one test for each molecule. Detection of single molecule by using AFM and EM, in which angular distributions or distance from end-to-end profiles are used, are usually analyzed for individual molecules by a sliding window method, resulting in narrower duration distributions because of correlation evaluation. All particles showing isotropic position distributions have been considered in this experiment to avoid bias in the measurements. In addition, the effects of

interactions between DNA strands and other molecules, such as proteins, can be studied. The researchers describe a sensitive method for determining how long DNA remains. By measuring the motion of gold nanoparticles tethered to the substrate by DNA molecules, experiments show that the position distributions show an obvious Gaussian character. Under the same environmental conditions, the mean duration of 45 monomers with different lengths was 35 nm with a 2.8 nm of mean standard error. This result is consistent with other results.

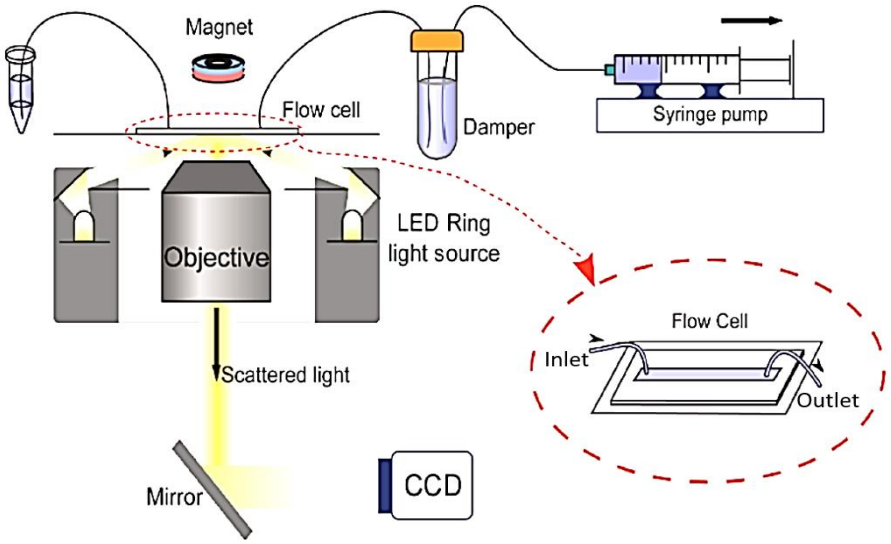


Figure 2. The principle of the developed DFM [3].

Lee et al. applied a simple DFM to quantitatively measure the thermal fluctuations of the micron beads attached to the DNA under fluid dynamics [3], as shown in Figure 2. In this work, researchers attached different lengths of DNA to beads, and measured the external force on the DNA. The results show an inverse correlation between thermal fluctuations and DNA length.

A multi-channel monomolecular flow stretching method that has better spatial resolution is proposed. In this method, to construct the DFM, a circular LED array lamp is installed around the objective lens of an inverted microscopy and placed under a sample table. In these conditions, no additional complex optical devices are required to prevent transmission of astigmatism. In addition, when using symmetrical dark-field lighting, the objective only collects uniform light scattered from the object. Therefore, the contrast and the signal-to-noise ratio can be improved. In this study, the researchers demonstrated DFM multiplex monomolecular flow stretching force spectroscopy, which can image microbeads attached to DNA. Uniform dark-field illumination of molecules stretched by hydrodynamics facilitates separation of DNA beads from surface potholes. The spatial resolution is further improved by using shorter DNA.

2.2. Protein detection

Markhali et al. proposed a large-scale parallel single-particle sensing method for interleukin-6 (IL-6) detection based on gold nanoparticle core-satellite formation [4]. This method is based on the localized surface plasmon resonance (LSPR) changes with core-satellite formation, leading to the observed colour change for core-satellite generation. In this method, gold nanoparticles with a hue value of 67 nm in thousandths were analysed by using Matlab to fix the nanoparticles with IL-6 as the target protein on the glass-covered surface before and after adding the reporter. The developed method shows some advantages. And it also can avoid the influence of non-specific adsorption, which is a problem for biosensing.

Nonspecific adsorption is a long-term and significant challenge for most sensors. When a non-target molecule is attached to the detected surface, background noise will be generated, resulting in non-specific adsorption, which will cause the signal to be covered by the background noise, making the signal difficult to distinguish [5,6]. This is a challenge, exacerbated as sensor sensitivity increases. This is particularly problematic for sensors with high sensitivity, where any small change produces a

signal. In this work, researchers used porous silicon photonic crystals for the first time to monitor enzyme activity, where a blue shift was observed through specific enzyme activity, rather than a red shift caused by specific adsorption. Plasma nanoparticles were previously demonstrated by the surface-enhanced Raman scattering (SERS) method, in which a single Raman dye (Rhodamine 6G) was detected. In this system, dye specific nanoparticles in the Raman hot zone have much greater signal strength than non-specific signals, so they can be distinguished. These two strategies suggest that a single nanoparticle sensor has an advantage in reducing the impact of non-specific adsorption in the configured analysis, but neither approach is comparable to what can be achieved with large-scale parallel single nanoparticle sensor compatible and integrated sensors that need to provide reliable quantitative information.

In previous studies, researchers have shown that the signals of single molecule from multiple plasma nanoparticles can be captured by the DFM. This method has the potential to be used in single particle detection. Because the dark-field sensing method is capable of providing quantitative information, this research focuses on the reason why the analysis signal of dark-field sensor is not sensitive to non-specific adsorption.

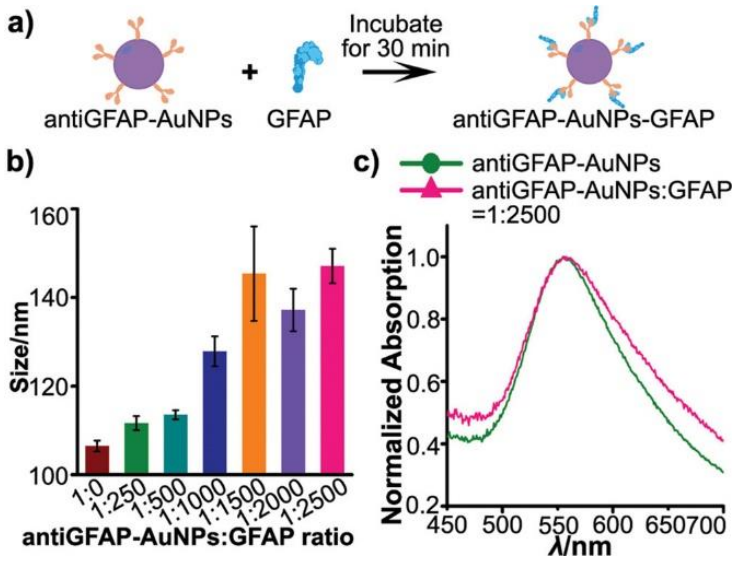


Figure 3. The characterization of the antiGFAP-AuNPs [8].

The proposed biosensor uses a DFM to obtain dark-field images of gold nanoparticles, and the formation of core satellite can result in a colour change for the scattered light. The research results show that the dark-field sensing method as an integrated sensor can provide a quantitative information based on the response of the whole surface. However, in order to understand the advantages of signals coming from many core nanoparticles bound to a single surface, one needs to study the signal changes of many nanoparticles at the level of a single nanoparticle. By plotting the positions of single nanoparticle in the images before and after adding the report, the colour offsets of multiple nanoparticles are detected.

Based on previous studies, the LSPR-based colorimetric sensing method was developed. When a single protein binding event binds the antibody nucleus and satellite nanoparticles together, the method can rapidly analyze the colour values of multiple nanoparticles simultaneously. The feasibility of the developed biosensing method was demonstrated by using cytokine IL-6. Transmission electron microscopy and DFM imaging show that the developed system can measure individual nanoparticle events.

The advantage of starting with a single nanoparticle is that the method is very tolerant of nonspecific adsorption. The results showed that the types of nonspecific adsorption did not occur. These experimental results were further confirmed by the computational study, where the 10 nm reporting nanoparticles produced a sufficiently large colour shift after the formation of the nuclear

satellite. It may be the subject of further research in this area in the future. The sensor is more robust and resistant to non-specific adsorption than a gold film with a continuous substrate.

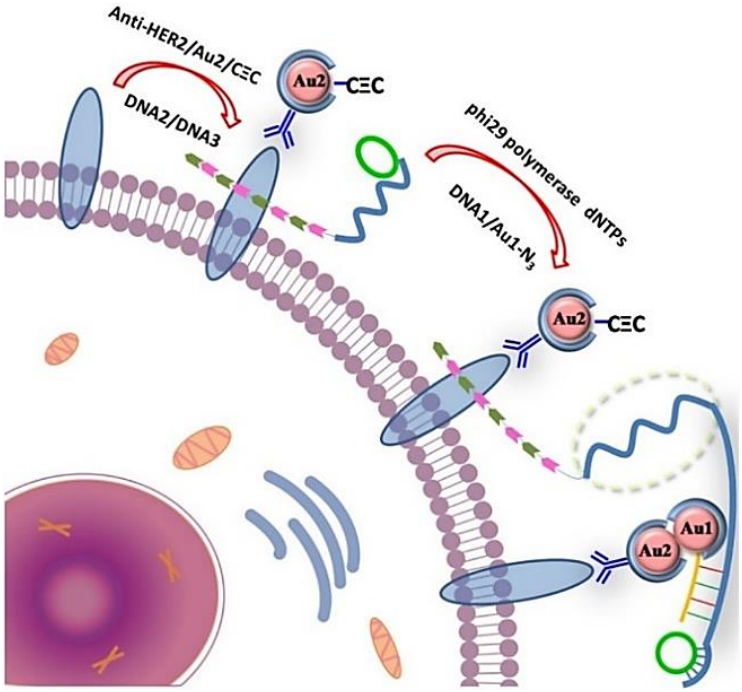


Figure 4. The principle of AuNPs nanoaggregates for HER2 protein detection on the surface of cell [9].

Peng et al. developed a new DFM for analysis of GFAP and peripheral blood mononuclear binding cells (PBMCs) [8]. As shown in Figure 3, the DFM developed an acquisition software specific anti-GFAP monoclonal antibody functionalized gold nanoparticles (antiGFAP-AuNPs) for rapid 3D imaging of PBMC with a customized written image as a contrast generation probe. Then, the algorithm was used to rapidly visualize and enumerate bound antiGFAP-AuNPs leukocytes from thousands of images. The developed method is to demonstrate the specific binding of GFAP on the PBMC surface. Then, the PMCS subgroup that binds to antiGFAP-AuNPs was identified as opening a new avenue for auxiliary studies of fluorescence imaging and DFM imaging in order to understand the relationship between traumatic brain injury and leukocyte species. Therefore, this study provides a rapid and ultra-sensitive method for the assessment of biomarkers after traumatic brain injury.

The immune cells play a key role for capturing a diverse of different antigens, which identifies the interaction between antigens and immune cells important not only for understanding immune responses, but also for early detection of disease. This strategy creates huge potential opportunities for assessing the severity of TBI at the single cell level. Of particular concern is not only the overall increase in immune cell-binding GFAP after TBI, but also the leukocyte binding distribution. This powerful strategy is expected to be used in the future to rapidly track TBI outcomes and assess treatment outcomes.

Guo et al. observed the phenomenon using a DFM based on the LSPR, a biological imaging technique that promises to enable no-damage, no-light bleaching [9], as shown in Figure 4. When asymmetrically modified AuNPs were used as probes, the nanoaggregates with stronger scattering efficiency were formed in the presence of the target. Firstly, Au1-N3 probe and Au2-C≡C probe were designed for the cycloaddition of azide and alkyne to form AuNP dimer [10,11]. Both probes were successfully used for detection of Cu^{2+} in rat serum. This concept is then applied to analyze proteins on cells, by modifying DNA and antibodies on probes. Both DNA1/Au1-N3 probe and anti-HER2/Au2-C≡C probe for HER2 protein were developed [12]. This unique design makes it easier to distinguish target signal from background noise, which can be applied to molecular diagnosis and cell imaging.

3. Conclusion

In short, DFM is a simple and effective microscopic technique. Almost any open field laboratory microscopy can be easily converted for dark-field illumination. By using this method, particles can be seen and the resolution is greatly improved compared with ordinary microscopy. DFM are also challenging to use. Special care must be taken when preparing specimens for DFM because features located above and below the focal plane scatter light and degrade image quality. The cleanliness of the slide is an important factor affecting the imaging, where fine debris will be illuminated and the observer will not be able to see the desired part. Other problems you may face when setting up a microscopy for dark field observation include: (1) There is insufficient illumination for the specimen to be viewed, or the specimen can be viewed in very weak light. The DFM requires a lot of light to image properly, since much of the light is blocked and conical. Therefore, it is necessary to adjust the lighting power, but it should be noted that some samples may not withstand a large amount of light exposure for a long time; (2) The slide has a dark spot in the center of the field of view, but the peripheral observation object is well lit and looks normal. This is usually caused by the condenser being improperly aligned or focused. This problem can be solved by centering and focusing the condenser. This solution works for most cases where the lighting looks uneven but the sample is clearly visible; (3) There is colour in the background, or the background is not uniform due to the existence of gray scale. This is usually due to the sample being too thick or the sample being installed in a contaminated medium. Reinstalling the sample should solve the problem.

4. Authors' Contributions

Qiuyi Tang completed the work design and article writing.

References

[1] Peng Fei Gao, Gang Lei, and Cheng Zhi Huang. Dark-Field Microscopy: Recent Advances in Accurate Analysis and Emerging Applications. Analytical Chemistry 2021, 93 (11), 4707 - 4726.

[2] Sanneke Brinkers, Heidelinde R.C. Dietrich, Frederik H. de Groote, lan T. Young, and Bernd Rieger. The persistence length of double stranded DNA determined using dark field tethered particle motion. The Journal of Chemical Physics 2009, 130, 215105.

[3] Ryanggeun Lee and Yongmoon Jeon. Single-Molecule DNA Flow-Stretching Force Spectroscopy with Higher Resolution Using Dark-Field Microscopy. Journal of the Korean Physical Society, 2019, 75 (4), 350 - 354.

[4] Bijan P. Markhali, Manish Sriram, Danielle T. Bennett, Parisa S. Khiabani, Sharmin Hoque, Richard D. Tilley, Padmavathy Bakthavathsalam, and Justin Gooding. Single particle detection of protein molecules using dark-field microscopy to avoid signals from nonspecific adsorption. Biosensors and Bioelectronics 2020, 169, 112612.

[5] Lichtenberg, J.Y., Ling, Y., Kim, S. Non-specific adsorption reduction methods in biosensing. Sensors 2019, 19 (11), 2488.

[6] Pan, S., Zhang, H., Liu, W., Wang, Y., Pang, W., Duan X. Biofouling removal and protein detection using a hypersonic resonator. ACS Sens. 2017, 2 (8), 1175 - 1183.

[7] Sriram, M., Markhali, B.P., Nicovich, P.R., Bennett, D.T., Reece, P.J., Brynn Hibbert, D., Tilley, R.D., Gaus, K., Vivekchand, S.R.C., Gooding, J.J. A rapid readout for many single plasmonic nanoparticles using dark-field microscopy and digital color analysis. Biosens Bioelectron. 2018a, 117, 530 - 536.

[8] Fei Peng, Sinyoung Jeong, German Gonzalez, Haley Marks, Alexander Ho, Emmanuel Roussakis, Petra B. Krauledat, Peter Hansen, and Conor L. Evans. Assessment of Glial Fibrillary Acidic Protein Binding to the Surface of Leukocytes with Dark-Field Imaging and Computational Analysis. Adv. Funct. Mater. 2021, 31, 2009229.

[9] Yingshu Guo, Fei Liu, Yinhua Hu, Xiaofei Zheng, Xiuping Cao, Yanxi Zhu, Xiaoru Zhang, Dongjiao Li, Zhenhua Zhang, and Si-kai Chen. Activated Plasmonic Nanoaggregates for Dark-Field in Situ Imaging for HER2 Protein Imaging on Cell Surfaces. Bioconjugate Chem. 2020, 31, 631 - 638.

[10] Baskin, J. M., Prescher, J. A., Laughlin, S. T., Agard, N. J., Chang, P. V., Miller, I. A., Lo, A., Codelli, J. A., and Bertozzi, C. R. Copper-free click chemistry for dynamic in vivo imaging. Proc. Natl. Acad. Sci. U. S. A. 2007, 104, 16793 - 16797.

[11] Wang, X., Liu, Y., Fan, X., Wang, J., Shu, W., Ngai, C., Zhang, H., Li, J., Zhang, G., Lin, J., and Chen, P. R. Copper-triggered bioorthogonal cleavage reactions for reversible protein and cell surface modifications. J. Am. Chem. Soc. 2019,141, 17133 - 17141.

[12] Zhu, Y., Chandra, P., and Shim, Y. B. Ultrasensitive and selective electrochemical diagnosis of breast cancer based on a hydrazine-Au nanoparticle-aptamer bioconjugate. Anal. Chem. 2013, 85, 1058 - 1064.