The applications of NSOM/QDs-based single-molecule in situ detection on cell membrane

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Abstract: Near-field scanning optical microscopy (NSOM) has been proved to be a useful nanotechnology tool for studying materials due to its highest optical resolution to break through the diffraction limit, but its applications in biomedical research are still limited. With the development of biology, medicine and chemistry, the methods to study life science in single molecule level are required. The high optical resolution property makes NSOM an ideal tool to study cellular ultrastructure, function, single biomolecule distribution and even the relationship of biomolecules on cell membrane. But the complicated natures of cell membranes or biologic molecules and the restriction of conventional organic fluorophores (low luminescence and photostability) make it hard for NSOM to generate high spatial resolution images. The development of quantum dots (QDs) overcome these technological barriers successfully. The combination of NSOM and QDs is recognized as a fascinating technology to study cell biology in single molecule level without injuring biological samples. In this chapter, we will introduce the basic principles of NSOM/QDs method, the recent advances of NSOM/QDs in single-molecule in situ detection on cell membrane and make an outlook on their application prospects.

Keywords: NSOM; quantum dots; single molecule; cell membrane

1. Introductions

The development of life science requires precise quantitation and accurate explanation of biological phenomenas which urge us to explore these phenomenas at molecular level. As the natural extension of molecular biology, molecule detection technology was developed to detect the behavior of biomolecules and to achieve molecular manipulation.

In recent years, nanotechnology is widely developed and makes a significant contribution to the advancement of life science and medicine. Cell membrane single-molecule detection is a new emerging research field due to the important role of membrane biomolecules in cellular transmembrane signaling pathways [1, 2]. A lot of works have demonstrated that cell membrane is made up of various nanosize and micrometer domains which primarily include caveolae [3], lipid rafts [4] and transient confinement zones [5]. To know how these microstructure domains behave in physical conditions would be helpful to understand the underlying mechanisms involved in cell membrane signal transduction [6]. The size of biological macromolecules and their clusters on cell surface is varying from several nanometers to hundreds of nanometers. The highest resolution of conventional optical microscopy, even the resolution of most widely used optical microscopy - confocal microscopy can just increase to 200nm due to the restrictions of optical diffraction limit. The complicated natures of cell membranes, small biologic molecules and the restrictions of optical diffraction limit make it difficult to achieve single-molecule in situ detection on cell membrane by conventional optical microscopy.

Along with the development of scanning probe technique, a new optical technique - near field optical technique comes out and breaks the optical diffraction limit revolutionarily. The development of near-field scanning optical technique makes high resolution optical microscopy possible. As a product, near-field scanning optical microscopy (NSOM) was first reported to a resolution higher than 50nm [7] and showed a potential application in cell and molecule detection [8].

2. Overview of NSOM

The fundamental principles of near-field can be described as following: The movement of probe interior electric charge will cause the change of the electromagnetic field around it and spread the electromagnetic field from the probe surface to free space. The probe surface field can be divided into near field area and far field area [7] as shown in Fig 1. The laser propagates through the sub-wavelength aperture on NSOM probe [9]. When a sample is close to the probe in near field, the area of the light spot on sample is just relevant to the aperture size so that the resolution of the NSOM doesn’t rely on the light wavelength.

NSOM is always made up of computer, optical system, high resolution image display management system, electronic control system and equipment detection system. When it’s working, the laser is coupled into the optical probe through the optical fiber. The probe has a sub-wavelength aperture on it and the probe is fixed on the piezoelectric ceramic tuning fork. The piezoelectric ceramic tuning fork can vibrate parallel to the sample by controlling the external voltage action on tuning fork. When the distance between probe and sample changes, the amplitude of tuning fork also changes.
due to the force change between probe and sample which also affects the voltage of tuning fork and finally makes it possible to monitor the distance between probe and sample. When the probe is scanning along with the surface of sample, there uses a feedback loop to keep the distance of probe and the sample surface. The feedback signals can reflect the height changes of sample, and finally get the surface morphology of sample. The probe of NSOM is very close to the sample surface which is just 1-10nm so that the laser from the sub-wavelength aperture on probe can irradiate on sample in near field. The transmitting beams from sample are collected by the detection system and finally form the optical images of sample after managing with the image management system and computer. If you want to detect the fluorescence signal of sample, there just needs an optical filter to be placed between microscope objectives and photomultiplier tubes to collect the fluorescence signal. The resolution of NSOM doesn’t dependent on the wavelength of incident light but is comparable to the size of the sub-wavelength aperture on probe, giving NSOM incomparable advantages compared with other optical technology.

**Fig.1.** The working principles of NSOM. (A) The principles of NSOM probe. The gray arrow in (A) indicated the movement of NSOM probe when it’s working. (B) The working systems of NSOM.

The appearance of NSOM not only provides scientists a useful tool to study the morphology and optical properties of materials [10, 11], but also provides scientists a powerful tool to do sing-molecule detection, such as DNA or biomacromolecule [12, 13], cell or bacteria imaging [14, 15, 16] and fluorescence resonance energy transfer detection of biomolecules [17].

The high resolution cell membrane single molecule imaging techniques were widely developed in recent years which include stimulated emission depletion microscopy (STED), photoactivated localization microscopy (PALM) and two-photon 4Pi microscope (4Pi). These techniques both break diffraction limit and show a high resolution in single molecule imaging. But the equipment of STED is too complicated and expensive so that only few groups mastered this technique. The equipment of PALM is also complicated and the time for imaging is too long (nearly 12h). Compared with these two techniques mentioned before, the resolution of 4Pi is much lower (about 100nm). It is worth noting that the topographical resolutions of these three techniques still both been restricted by the diffraction limit. So we can just get fluorescence information from these techniques. The most outstanding property of NSOM is that it combines the high resolution topography and high resolution fluorescence images.

Additionally, NSOM is regarded as best microscope to study membrane proteins through detecting the fluorescent probe capped with protein[18]. Combined with fluorescent dyes, NSOM was used to detect the distribution of emissive molecules in human ocular lipofuscin granules, and showed a higher resolution than confocal microscopy [19]. It was also used to detect the distribution of LFA-1(αL/β2) molecules on mouse embryonal fibroblast cell membrane [20], proving the potential that NSOM could be used to realize single-molecule detection on cell membrane.

3. **Overview of quantum dots**

Fluorescent dyes and fluorescent proteins are the main biomedical markers which are widely used today. The discovery and development of green fluorescent protein molecules contributed to a new method to study the structure, conformation and function of proteins. The discovery of organic fluorescence probe also promoted the development of cellular molecular markers.

These traditional fluorescent dyes and fluorescent proteins still play an important role in life science. NSOM combined with some fluorescent materials were used for imaging in previous works [21, 22, 23], but their weak fluorescent intensity and light stability hindered their further applications. In 1998, quantum dots (QDs) were first...
QDs are fluorescence semiconductor nanocrystal which are mainly made up of II B/VI A (such as CdSe) or III A/ V A (such as GaAs) elements and have stable and soluble properties, ranging from 1-100 nm. Different QDs have different emitting wavelength, even they are made up of same material and components, their sizes will affect their emitting wavelengths a lot. But there is an outstanding characteristic that all QDs will be excited by lights whose wavelengths are 10 nm smaller or even much smaller than their emitting wavelengths [28]. Compared with traditional fluorescent dyes and fluorescent proteins, QDs have a lot of advantages:

1. All QDs have wide wavelength for excitation, with narrow wavelength for emission. This property makes it possible that using only one kind of exciting light to excite different kinds of QDs to obtain several different emitting lights. But for traditional fluorescent dyes and fluorescent proteins, different kinds of fluorescent materials need different lights to excite due to the narrow wavelength range for excitation.

2. The narrow emitting wavelengths of QDs are symmetrical while nearly all traditional fluorescent dyes and fluorescent proteins have nonsymmetrical emitting wavelengths and have overlapped emission spectrum. This property will pose interfere with other fluorescent materials when several kinds of fluorescent materials are used for detection.

3. The fluorescence intensity and stability of QDs are much higher than that of traditional fluorescent dyes and fluorescent proteins which make it possible to do long time observation after samples were labeled with QDs.

4. QDs have very good biocompatibility. After capped with biomolecules, QDs showed very good fluorescent property but low cytotoxicity[29].

For these advantages, QDs are widely developed now to be new fluorescent probe for cell biomolecules in situ detection [30, 31], showing a more and more important role in biomedicine.

### 4. Overview of NSOM/QDs method

In NSOM/QDs method, QDs are always coupled with streptavidin while the antibody of membrane biomolecules is linked with biotin. The basic principles of NSOM/QDs method are expressed in Figure 2. Antigens are distributed on cell membrane according to certain rules. When biotinylated antibodies are added for incubation with cells, these antibodies will be attracted by antigens on cell membrane and will link with each other due to the specific forces between them. Once QDs-streptavidins are added, the streptavidins will contact with the biotins on antibodies specifically. When this QDs-antibody-antigen system is accomplished, the location of QDs could reflect the location and distribution of antigens exactly. The detection process is started by a laser which could excite QDs, the detection system will collect the fluorescent signal emitted by QDs and topography information. Combined with NSOM topographic and fluorescence images, the location and distribution of antigens on cell membrane will be presented with high resolution. Another method to constitute QDs- antibody- antigen system is double antibody labeling method. The first antibody is used to contact antigen and the second antibody which coupled with QDs is used for connecting the first antibody.

The general staining procedure for NSOM/QDs method is biotin- streptomycin method: cells grown on the sterile cover slip are washed according to PBS buffer, fixed by 2% formaldehyde and washed triple by PBS before further staining. The cells are blocked with 1% bovine serum albumin and then incubated with biotinylated antibodies. After incubation, cells are washed to remove the unbounded antibodies and then incubated with QDs-streptavidin [32]. Additionally, double antibody method is also a conventional method used for NSOM/QDs method. For example, if someone wants to detect human CD 3 molecules on T cells by NSOM/QDS method using double antibody labeling method. The fixed cells should be incubated with rabbit anti-human CD3 to label CD3 molecules, followed by anti-rabbit IgG (H+L) conjugated QDs staining for connecting rabbit anti-human CD3 [33]. After washed with PBS, NSOM can be used to obtain the topographic and fluorescence images of these samples. When there are two different molecules need to be labeled, these two methods must both be used.
5. Applications of NSOM/QDs method in single-molecule in situ detection

5.1. Single-molecule imaging on cell membrane

NSOM/QDs method was first reported by our research team in 2006 at Asian Nanoscience and Nanotechnology Conference [34] and showed an abroad application prospect in cell membrane single-molecule detection. After that, we cooperated with a team from University of Illinois and focused on the applications of NSOM/QDs method in life science. Our further results were then published in BLOOD and featured on the cover page [35]. In the previous works, nanoscale imaging of an in vivo antigen-specific T-cell immune response has not been reported. In this study, NSOM/QDs method was used to do immuno-fluorescence imaging of antigen-specific T-cell receptor (TCR) response in an in vivo model of clonal T-cell expansion. The NSOM/QDs system provided nanoscale images of VγVδ TCR on the membrane of nonstimulated Vγ2Vδ2 T cells which was shown in Figure 3. The images obtained in this work absolutely broke the diffraction limit and got a best-optical resolution (~50 nm) for nanoscale imaging. The short arrow in Figure 3C showed an approximately 41-nm fluorescence dot representing a 1-QDs–bound TCR dot which might be the indicator of single TCR, and the long arrow shows an approximately 80-nm fluorescence dot possibly corresponding to 2-QDs-bound TCR cluster. The higher-magnification imaging shows immuno-fluorescence images of individual 1× approximately 50-nm TCR dots (short arrows), 2× approximately 50-nm TCR cluster (long arrow), and more than 2× approximately 50-nm TCR clusters (dotted circles). Mean FWHM diameters of the QD-Ab-bound TCR were 53.7 plus or minus 18.9 nm (mean SD), with approximately 50-nm dots being predominant. This work confirmed that using NSOM/QDs method to do cell membrane single-molecule in situ detection was not only feasible in theory but also realizable in reality. This work provided new nanoscale insight into the in vivo T-cell immune response and provided a new cell membrane single-molecule in situ detection method.
5.2. The better resolution of NSOM/QDs method in Single-molecule imaging

NSOM/QDs method was then used to study the nanoscale organization of CD44 molecules of fixed mesenchymal stem cells (MSCs) in air [32]. Confocal microscopy is the most widely used tool for fluorescence imaging. The resolution of NSOM and confocal microscopy were examined and shown in Figure 4. Apparently, the resolution is too low to resolve individual CD44 domains for confocal microscopy, making it impossible to map the nanoscale distribution of membrane protein. The images obtained by NSOM could show individually resolved domains with a resolution of <50 nm, showing the higher spatial resolution of NSOM. Quantitatively statistics of fluorescence intensity and size were also used to investigate the spatial organization of CD44 molecules. The photons of a single QD are approximately 0.5 kCounts while the photons detected per domain had a median value of about 1.5 kCounts which demonstrated a wide spread in the number of CD44 molecules per spot. The size-distribution histogram was shown in Figure 4G, the spot sizes ranged from less than 200 nm to almost 600 nm, with a median value about 275 nm while the smallest fluorescence spot is about 50 nm, similar with the probe aperture size of NSOM.
5.3. The better fluorescence properties of QDs in Single-molecule imaging

The fluorescence intensity changes of QDs and the widest used fluorescent dye-FITC was examined by NSOM [34]. The results exhibited that the fluorescence intensity of FITC was much lower than that of QDs. The fluorescence intensity of FITC faded quickly and became undetectable after continuously illumination, whereas the fluorescence intensity of QDs showed no obviously changes even at longer time. These results exhibited the excellent fluorescence intensity and phtostability of QDs and so that QDs may be employed as the long-term monitoring cell marker instead of common dyes.

5.4. Fluorescence-topographic images obtained by NSOM/QDs method

The NSOM 3D-topography images are very useful in confirming the location of membrane biomolecules. As we know, there are membrane fluctuations on T cell membrane which is closely related to the rearrangement of actin and microtubule cytoskeleton [36]. The traditional optical microscopy doesn’t have a well enough resolution to detect cell membrane fluctuations so that the distribution of membrane biomolecules on the membrane fluctuations (in the valley or in the peak) can’t be clearly observed. So far, NSOM is the only technique that combines nanometric optical resolution with simultaneous nanoscale topographic information. Figure 5 showed the fluorescence-topographic images of GM1/CD59 nano/microclusters detected by NSOM. We can get the distribution information of GM1/CD59 molecules on cell membrane directly through the fluorescence-topographic images [37]. NSOM/QDs method is the best technology can be used to detect the distribution of cell membrane biomolecules due to its nanoscale fluorescence-topographic images.
5.5. Dual molecules detection simultaneously using NSOM/QDs method

Another important advantage of NSOM/QDs method is that it can provide two-color molecular staining using only one exciting light. Such as in Figure 6, QDs605 and QDs655 were used to stain CD3 molecules and CD4 molecules, respectively. Because there were two different molecules need to be labeled, biotin-streptomycin method was used for CD4 labeling and double antibody method was used for CD3 labeling. The images obtained by NSOM showed the distribution of CD3 and CD4 molecules. Furthermore, the merged NSOM images showed that CD3 and CD4 were co-clustering within the same nano-domains, suggesting that the TCR/CD3 interacted with CD4 co-receptor after anti-CD3 Ab stimulation and formed co-clustering nanostructures for sustained T cell activation. This directly showed the distributional relationship between TCR/CD3 and CD4 molecules after anti-CD3 Ab stimulation [33]. This implied that NSOM/QDs-based dual-fluorescence technology could provide us a very effective tool to study the relationship of two different biomolecules on cell membrane.

5.6. Dual-fluorescence-topographic images obtained by NSOM/QDs method

Dual-fluorescence images and fluorescence-topographic images could show the information of the relationship of two different biomolecules and the locations of biomolecules on cell membrane, respectively. Combining these images, more delicate distribution relationships of biomolecules on cell membrane are able to be obtained.

NSOM/QDs-based fluorescence-topographic images can directly reveal the nano-spatial peak-valley polarities of biomolecules on cell-membrane fluctuations. As shown in Figure 7[38], most of CD69 nano-domains were polarized predominantly in the peak of the cell-membrane fluctuations while CD71 nano-domains didn’t co-localize with CD69 nano-domains but mainly located in the valley of the cell-membrane fluctuations. The different locations of CD69 and CD71 molecules implicate their different biological activities during the T-cell activation.
This NSOM/QDs-based dual-fluorescence–topographic method makes it possible to directly image the exact distribution of cell membrane biomolecules and the relationship between these biomolecules on cell-membrane fluctuations.

![Figure 6](image)

**Figure 6.** The NSOM dual color images of one representative of the anti-CD3 Ab-stimulated T-cells. The upper (whole-cell images) and middle (zoom images) panels show nano-clustering of CD3 (dark blue) and CD4 (red) as well as co-clustering of CD3-CD4 (overlay pink) forming nano-domains or micro-domains on cell surface of an activated T-cell, as illustrated by white arrows [33].

5.7. The combination of NSOM/QDs method and confocal microscopy

Additionally, there are also methods which combine NSOM with other microscopy to detect biological samples. NSOM can just provide the distribution of biomolecules on cell membrane which is a weakness of NSOM. But this property can also be recognized as an advantage because the fluorescent signals obtained by NSOM are all from conclusive membrane biomolecules while the fluorescent molecules inside the cell can’t be detected. So NSOM also can be used to confirm that are biomolecules are located on membrane or not. Another important optical microscopy-confocal microscopy can provide the fluorescent images of intracellular molecules, but the resolution of confocal microscopy is lower than NSOM. Combined with confocal microscopy, NSOM/QDs method could provide more information of membrane biomolecules. Figure 8 showed the NSOM and confocal microscopy images of IL-22 and CD4 molecules on CD4 T cells [39]. The QDs labeled cells imaged by confocal microscopy indicated that both CD4 and IL-22 mainly distributed on cell membrane while nearly no fluorescence were detected in cytoplasm(Figure 8A). These confocal images showed that IL-22 on the surface of spleen T effectors localized as large capping domains, but couldn’t confer high-resolution nanoscale imaging of molecular details and couldn’t provide us the exact distribution of these molecules on cell membrane. NSOM images reported here had a higher resolution and showed that IL-22 cytokine proteins engaged themselves as 100–200nm nanoclusters or 300–600nm high-density nanodomains on outer membrane while CD4 molecules randomly distributed with no obvious nanodomains(Figure 8B). The formation of these IL-22 nanoclusters/ nanodomains on membrane suggested that large amounts of IL-22 were expressed on T cell membrane and actively involved in immune responses during active M. tuberculosis infection. Simultaneously, the IL-22 nanoclusters were seldom colocalized with CD4 molecules on cell membrane.
Figure 7. NSOM/QDs-based fluorescence–topographic images about the distribution of CD69 and CD71 molecules on T cell membrane. Upper panels showed 3-D images of T cell topography (left), CD69 fluorescence–topographic fusion images (right); lower panels showed CD71 fluorescence–topographic fusion images (left), dual color of CD69 and CD71 fluorescence–topographic fusion image (right) [38].

5.8. The combination of NSOM/QDs method and AFM

It is worth noting that AFM has emerged as a powerful tool for obtaining ultrastructural details of biological samples with high resolution and for measuring the forces between single biomolecule on cell membrane and modified tip over the past decades [40]. AFM was used to image biological samples combined with NSOM [41], but this work was just restricted for topography scanning. In another study, AFM force spectroscopy and NSOM/QDs method were both used to examine CD69 molecules expressed on PHA-activated human CD4⁺ T cell membrane [42]. Using AFM-functionalized tips, the CD69 antigen-antibody binding forces at the single-molecule level were measured. These detectable force spots on the membrane area could also illustrate the distribution of CD69 antigens on the CD4⁺ T cell. The CD69 nanoclusters on membrane of CD4⁺ T cells were also imaged by NSOM/QDs method and were consistent with what were seen by AFM force-binding analyses. As shown in Fig 9 and Fig 10, the NSOM/QDs image showed the distribution of 80–200 nm CD69 nanoclusters on a representative PHA-activated CD4⁺ T cell membrane. In addition, the adhesion force between CD69 Ab-functionalized tip and cell membrane indicated that few CD69 molecules were detectable on the cell-surface of the resting CD4⁺ T cell while many CD69 molecules were repeatedly detectable on the cell-surface of the representative PHA-activated CD4⁺ T cell. Combining these two single-molecule detection technologies, the ultrastructural details and nano-spatial distribution or location of CD69 expression on activated T cells were clearly imaged.

6. Future Prospects and Challenges

NSOM is developed by the traditional optical microscope and overcomes the diffraction limit to provide high optical resolutions. Although the resolution of NSOM is still low compared with transmission electron microscope and scanning tunnel microscope, the no damages and non invasions properties to biological samples make it widely used in biomedical or biomaterial fields. These outstanding properties also make it an ideal tool for single-molecule imaging and for the activities detection between biomolecules. The emergence of NSOM/QDs method makes a breakthrough in the field of single-molecule in situ detection. In this article, we have demonstrated the research advantage of NSOM/QDs method in single-molecule in situ detection on cell membrane and introduced the progress of NSOM/QDs method. This method was applied to do in situ detection of several cell membrane molecules, such as the membrane...
proteins, lipid rafts and cytokines. For further study, these results have a powerful stimulative effect on the application of NSOM/QDs method in life science and provide a new perspective of the relationship between the distribution and function of cell membrane molecules.

**Figure 8.** (A) Confocal microscopic images show that membrane-bound IL-22 formed capping domains on CD4 T cells after in vivo *M*. *tuberculosis* infection. Scale bars: 5 μm. (B) Representative NSOM/QDS-based nanoscale images. Lower panels, Show the enlarged images derived from rescanning the dashed squares in the upper panels. The scale bars for the upper and lower panels are 3.214 μm and 385 nm, respectively. (C) Upper panel, A representative fluorescent intensity profile of an IL-22 nanocluster. Lower panel, Histogram graph shows the frequency of different sizes of FWHM of IL-22 nanoclusters on the CD4+ T cells. [39]. (D) Upper panel, A representative fluorescence intensity profile of a CD4 nanocluster. Lower panel, Histogram graph shows the frequency of different sizes of FWHM of CD4 nanoclusters or nanodomains on the CD4+ T cells.
Figure 9. There were no fluorescence signal in the resting CD\textsuperscript{4}T cells (A), whereas a large number distinct non-uniform distribution fluorescence spots emerged in the fluorescence image of the CD\textsuperscript{4}T cell activated 24 h with PHA (B). A representative single fluorescence dot of QD-stained CD69 was marked by a cross-line in (C), and indicated as 82 nmFWHM by the NSOM software analysis (D). [42].

Figure 10. High-resolution image of a cell-surface area of (A) a representative unstimulated CD\textsuperscript{4}T cell and (E) PHA-activated CD\textsuperscript{4}T cell. (B) and (F) Adhesion force map of the same cell-surface area shown in (A) and (B), respectively. Adhesion force histogram recorded (n=512) with a CD69 Ab-functionalized tip of (C) unstimulated CD\textsuperscript{4}T cell and (G) PHA-activated CD\textsuperscript{4}T cell. Typical representative force–distance curve measured on (D) the resting CD\textsuperscript{4}T cell and (H) PHA-activated CD\textsuperscript{4}T cell. [42].
The NSOM/QDs method used in single-molecule in situ detection on cell membrane is an interdisciplinary technology which contains optics, nanotechnology, chemistry, cell biology, biomedicine and immunology. For biologists, immunologists and chemists, the complicated NSOM system is too difficult to understand and operate. But for opticians and nano experts, the biological and chemical phenomenon is also rarely understood. So there are only few groups who have mastered the NSOM/QDs technique for membrane single-molecule in situ detection. In order to expand the application of NSOM/QDs method for cell membrane single-molecule in situ detection, the simplified of NSOM operation system should be continued and the stability of NSOM system remains to be strengthened. In addition, more staining methods should be developed for triple-fluorescence or even quadruple-fluorescence staining in NSOM/QDs method. At last, how to image live cells in the liquid by NSOM/QDs method is also a critical issue needs to be resolved. We believe that with the maturity of equipment and technology, NSOM/QDs method will promote biology to micro field more thoroughly and make more innovative achievements revolutionarily.

References


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