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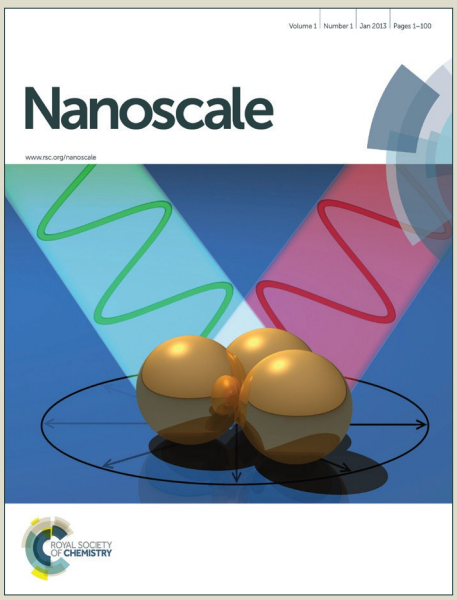

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ARTICLE

Enzyme Catalysis Enhanced Dark-field Imaging as a Novel Immunohistochemical Method

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The conventional immunohistochemistry is limited to subjective judgment based on experiences and thus it is clinically required to develop quantitative immunohistochemical detection. 3, 3'-diaminobenzidin (DAB) aggregates, a kind of staining product formed by conventional immunohistochemistry, were found to have a special optical property of dark-field imaging for the first time, and the mechanism was explored. On this basis, a novel immunohistochemical method for detecting HER2 overexpressed in breast cancer based on dark-field imaging has been established, and the quantitative analysis standard and relevant software for measuring the scattering intensity has been developed. In order to achieve more sensitive detection, the HRP (horseradish peroxidase)-labeled secondary antibodies conjugated gold nanoparticles were constructed as nanoprobes to load more HRP enzymes, resulting in an enhanced DAB deposition as dark-field label, as demonstrated. Simultaneously, gold nanoparticles also act as a synergistically enhanced agent due to their mimic enzyme catalysis and dark-field scattering properties.

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Introduction

Recently, dark-field microscopy becomes a hot characterization method in biomedicine due to its high resolution and sensitivity. As functional nanomaterial, gold nanoparticles have received a great deal of interest because of its competitive properties, such as high scattering cross section and resistance to photobleaching[1-7]. Gold nanoparticles with a diameter of 60 nm have a 10^5 times scattering intensity of fluorescent molecules[8, 9], and when it comes to 70 nm, the scattering intensity can be stronger than same-sized polystyrene microspheres by orders of magnitude[1]. Due to the unique optical properties, gold nanoparticles are widely used as light scattering agents[1-3, 5, 10-13]. El–Sayed[10] and Huang[2] respectively realized both cancer cell dark-field imaging and photothermal therapy in the near-infrared region at the same time using gold nanorods. Labeled by anti-EGFR-Au conjugates, the malignant cells could be visualized and diagnosed from the normal ones and selectively destroyed without harming the surrounding nonmalignant cells. With the improvement of dark-field imaging sensitivity, it is even possible to probe recognition binding events at the single-nanoparticle level[14, 15]. Raschke et al[15] proposed a real-time biomolecular recognition based on biotin-streptavidin affinity using light scattering spectroscopy of single gold nanoparticles. Besides various shapes of gold nanoparticles, recent researches on dark-field scattering are mainly focused on metal and metallic oxide nanomaterials, such as silver[16, 17], TiO_2[18] and iron oxide magnetic nanoparticles[19], few researches about organic materials were carried out.

For a long time, immunohistochemical method based on the specific binding of antibody and antigen serves as one of the most common tools in cancer detection due to its competitive advantages in aspects of simple and low-cost. The molecules to be detected are labeled by the primary and secondary antibodies sequentially to form immunocomplex, and the results are usually exhibited through color reactions, such as HRP-catalyzed DAB deposition reaction. However, conventional immunohistochemical method relies too heavily on experiences and subjective judgment to realize quantitatively detection. So it is urgently required to develop new techniques or to excavate out new functions from conventional methods applied in clinical diagnosis, just like new uses for old drugs.

Here we found that the breast tumor sections could be diagnosed by the significantly enhanced scattered light in dark-field after 3, 3'-diaminobenzidin (DAB) staining. We explored the mechanism and demonstrated that DAB deposition had a special optical property of dark-field imaging. On this basis, a direct dark-field imaging method based on HRP-catalyzed DAB deposition for the HER2 overexpressed breast tumor sections has been established, and the quantitative analysis standard and relevant software for measuring the scattering intensity has been developed. As a result, 114 cases of breast tumor sections with different expression levels of HER2 were successfully detected with a sensitivity of 96.70% and a specificity of 95.65%. In order to achieve more sensitive detection, gold nanoparticles were used to conjugate a large amount of HRP-labeled secondary antibodies to increase the quantity of HRP enzymes, resulting in an enhanced DAB deposition as dark-field label. Furthermore, gold nanoparticles also strengthened the catalyzed position of DAB through their peroxidase-like activity, as reported widely[20, 21]. Simultaneously, gold nanoparticles also act as a synergistically enhanced agent due to their dark-field scattering properties.

Results and discussion

Dark-field imaging based on HRP-catalyzed DAB deposition.

DAB is one of the most common tools for conducting color reactions in immunohistochemical method. The immunocomplex formed by antibodies and the molecules to be detected can be exhibited as brown deposition through DAB staining. During the DAB deposition reaction, the amino groups on side chains of DAB were oxidized by H2O2 under the catalysis of HRP or other peroxidase. Through repeated oxidative ring closing and polymerization, the insoluble brown phenazine polymer (PDAB) was formed as the deposition reaction products[22, 23] (Figure1a).

As observed under SEM, the breast cancer sections (Figure 1b) and cells of BT474 (Figure 1d) with HER2 overexpressed became much rougher than those without DAB staining (Figure 1c and e) due to the floccule produced in DAB deposition. The DAB oxidation products obtained directly from HRP-catalyzed DAB-H2O2

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reaction system were further evaluated by SEM, TEM and electron diffraction, showing obvious particulate morphology and amorphous structure (See Supplementary Figure 1a, b and c).

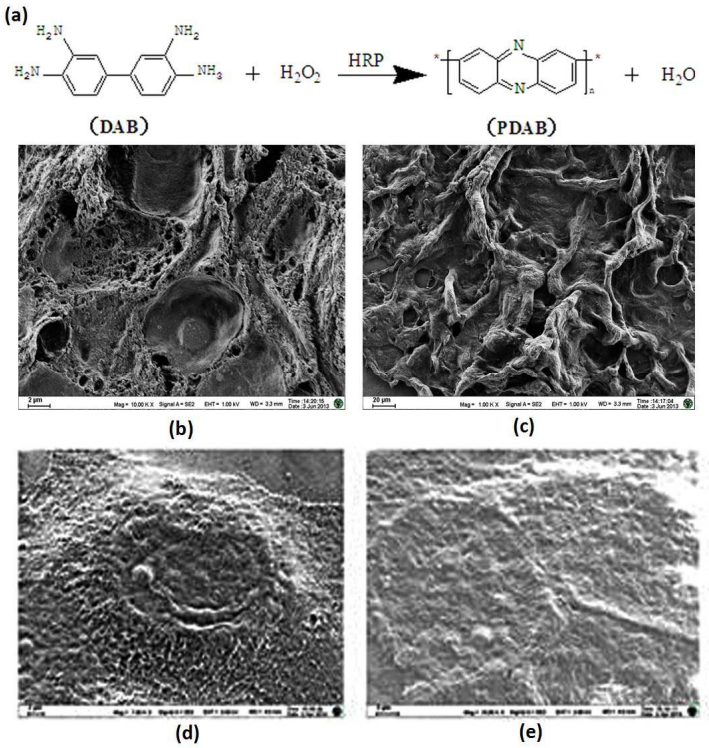

Figure 1. (a) DAB deposition reaction pathway. (b, c, d, e) SEM images of DAB oxidation products on breast tumour samples. Breast tumor sections after (b) and before (c) immunohistochemical stained with DAB. SEM images of breast cancer cell of BT474 (with HER2 overexpression) after (d) and before (e) immunohistochemical stained with DAB.

The breast cancer sections with strong positivity would bind more HRP labeled antibodies to catalyze and produce more particulate aggregates of oxidation products. According to the Mie scattering law, larger particles scattered more strongly and preferred to scatter longer wavelength of light. The situation was simulated by dielectric spheres of different radius (See Supplementary Figure 1d). When the expression level of HER2 was low, the DAB oxidation products were small and trend to scatter yellow-green light at short wavelength. When the expression level of HER2 was high, there would be more oxidation products which were more likely to form bigger aggregates and scatter red light at long wavelength. This proved feasibility of establishing the quantitative analysis method and the relevant software for breast tumor sections.

Based on these studies, PDAB seemed to have great potential in dark-field scattering, so that we conducted a series of experiments to explore the possibility of DAB dark-field imaging. 114 cases of breast tumor sections with four different expression levels of HER2 were used in the experiment and high-contrast images of dark-field were obtained by DAB deposition reaction. The scattering intensity of dark-field images was increased with the positive degrees, which was agreed well with the distribution of HER2 (Figure 2). Analyzed the results in Supplementary Table 1, the sensitivity of dark-field imaging based on conventional immunohistochemical DAB staining for breast cancer sections was 96.70%, the specificity was 95.65%, the misdiagnosis rate was 4.35%, the missed diagnosis rate was 3.30%, the positive predictive value was 98.88%, the negative predictive value was 95.65% and the Jordan index was 0.92, which proved that the detection method and the relevant software had a satisfactory reliability and accuracy. Besides that, the detection method have also been proved to be applicable to malignant lymphoma sections with CD20 overexpressed (See Supplementary Table 2).

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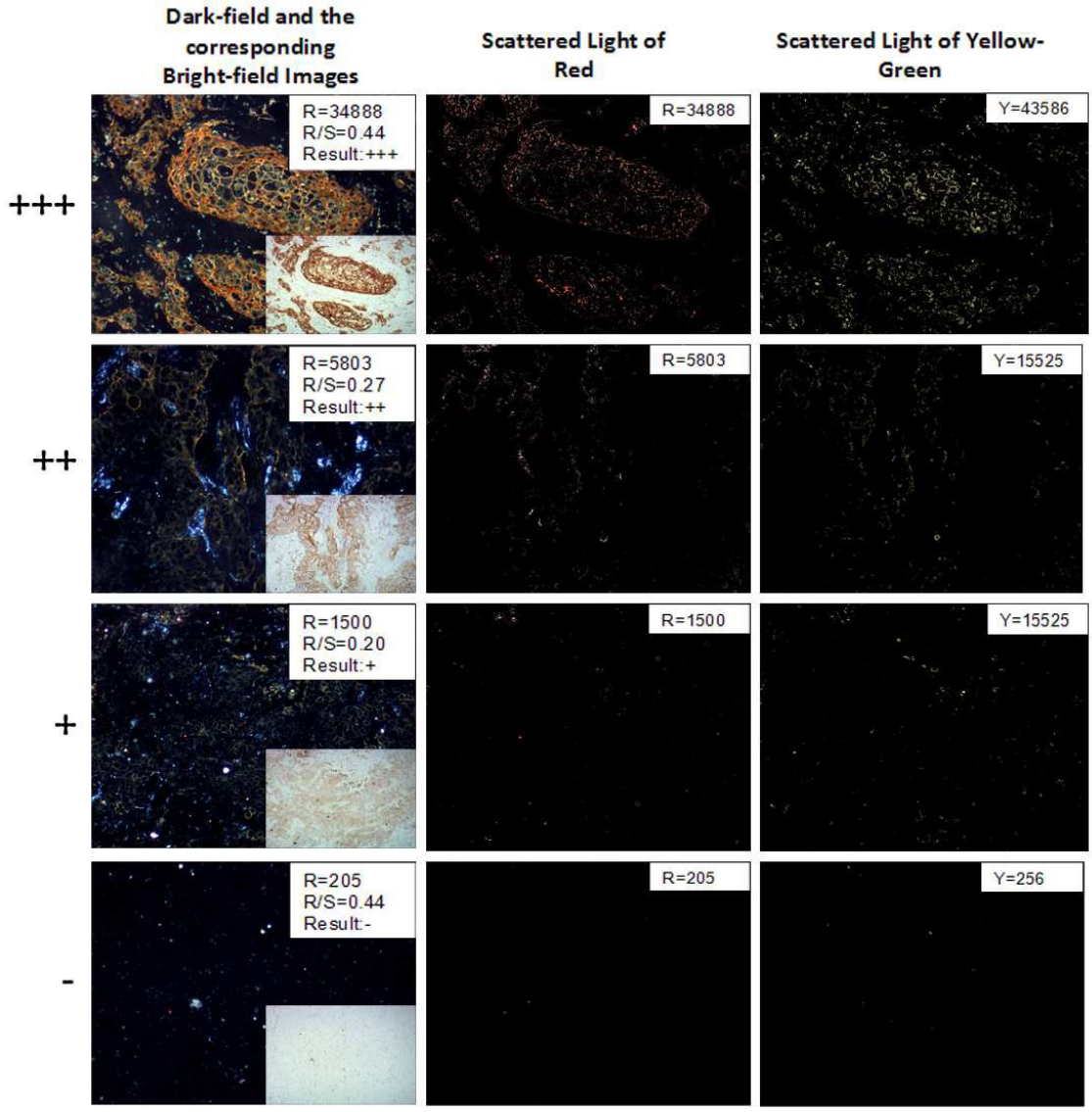

Figure 2. Analysis results of dark-field images(100×) given by DF-Analysis (the name of established dark-field analysis software). Breast cancer sections of different positive degrees were analyzed by dark-field imaging based on conventional immunohistochemical DAB staining. Color of dark-field image was segmented into red and yellow-green, and calculated separately.

The quantitative analysis method and software based on the scattering property of DAB deposition.

Conventional immunohistochemical technique was based on manually measuring, which cannot realize quantitatively detection and relies too heavily on the experiences and subjective judgment. So we developed a quantitative classification software to read the detection results instead of human eyes.

After performing the statistical analysis among 114 cases, we found that the breast cancer sections with different expression levels of HER2 had different scattering features. Strongly positive samples trended to scatter red light and weakly positive ones trended to scatter yellow-green light. The negative samples could not be successfully labeled and thus hardly scatted light in dark-field.

On this basis, the quantitative analysis method and relevant software DF-Analysis for quantifying the scattered light from the breast cancer sections stained by DAB has been developed. The breast cancer sections were dealt with conventional immunohistochemical method followed by performing statistical analysis using DF-Analysis (Figure 2), which worked by the following rules: ①Pick the color (R: red, Y: yellow-green) of the scattering images.

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②Analyze the scattering area, and calculate the ratio of the red area (R) and the sum of scattering area (S=R+Y). ③Compare the value of R and R/S with the standard ranges of different expression levels. When R<400, the result is "-". When 400<R<10000 and R/S<0.26, the result is "+". When 400<R<10000 and 0.26< R/S <0.35, the result is "++". When 400<R<10000 and R/S >0.35, the result is "+++". ④ Output the results and the processed images.

HRP and gold nanoparticle synergistically enhanced dark-field imaging for breast tumor sections.

Since the enzyme-like activity of nanomaterials has been found, gold nanoparticles have received a great deal of interest in biological and chemical detection due to their catalytic activity. In 2010, Chunying Chen[20] and coworkers established an enzyme linked immunosorbent assay (ELISA) for the detection of mouse interleukin 2 (IL-2) based on Au@Pt nanostructures, which were constructed using gold nanorods coated with a shell composed of Pt nanodots and exhibited intrinsic oxidase-like, peroxidase-like and catalase-like activity. In their later work[21], electron spin resonance spectroscopy (ESR) was used to investigate the catalytic activity of gold nanoparticles and gold nanoparticles were found to be able to catalyze the decomposition hydrogen peroxide, accompanied by the formation of hydroxyl radicals at lower pH and oxygen at higher pH. As a result of the mimic enzyme catalysis property of gold nanoparticles, we tried to use the Herceptin-Au nanoprobes labeled on the breast tumor sections which overexpressed HER2, to catalyze DAB deposition reaction directly. However, due to the limited catalytic activity of Herceptin-Au nanoprobes as shown in Supplementary Figure 2a, where antibody and BSA conjugation blocks the active sites on surface of gold nanoparticles, stray light of background was heavy and the bright-field images hardly showed obvious gradient (See Supplementary Figure 2b).

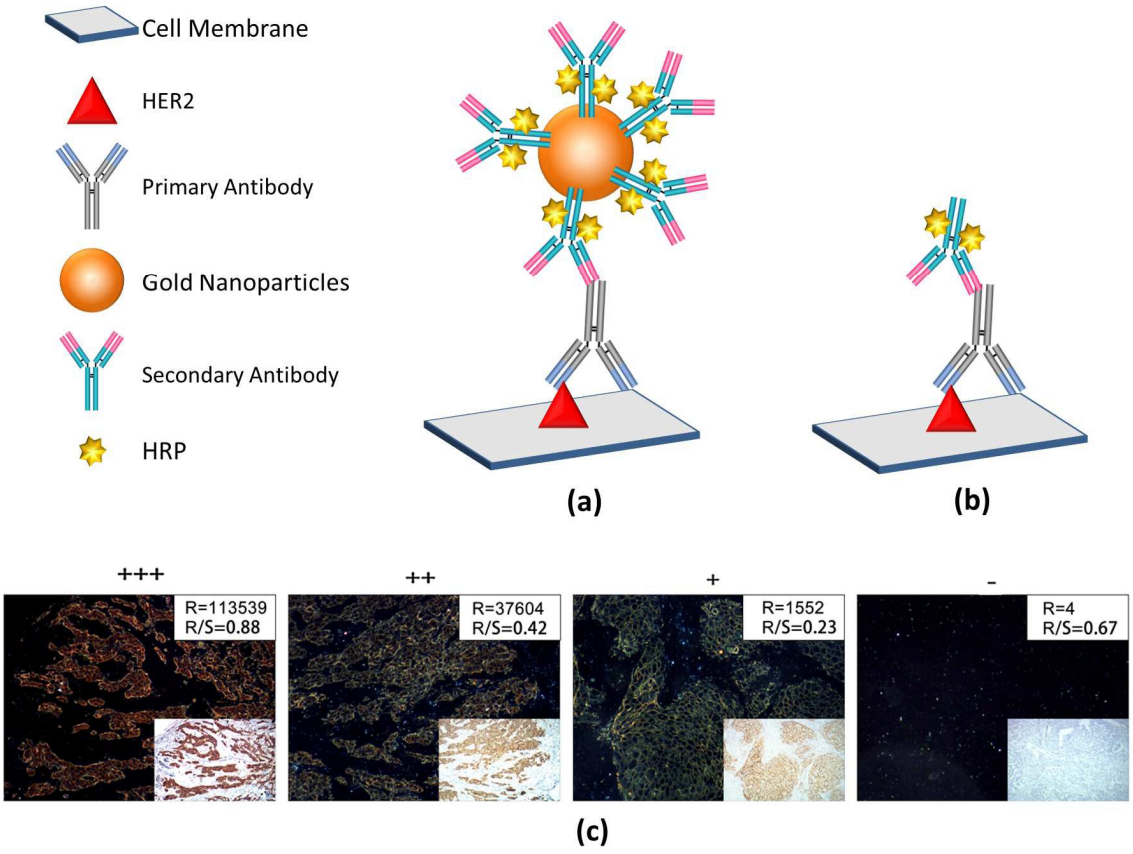

Figure 3.(a) Principle of synergistically enhanced dark-field method based on HRP-AbII-Au nanoprobes. (b) Principle of conventional immunohistochemical method.(c) Dark and the corresponding bright field images (100×) of breast tumor sections with varying degrees of HER2 analyzed by synergistically enhanced immunohistochemical method.

To obtain a higher-efficient nanoprobe, we established the synergistically enhanced immunohistochemical method by combining this nano-detection technology with the conventional peroxidase of HRP and realized the

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enlargement of signal. For conventional immunohistochemical method, HRP labeled secondary antibodies were used to catalyze color reaction with DAB as chromogenic substrate in the presence of hydrogen peroxide. When it came to this enhanced immunohistochemical method, a large amount of HRP labeled secondary antibodies were conjugated on one gold nanoparticle (Figure 3a), so that the HER2 could be labeled by much more HRP than conventional detection (Figure 3b), leading to an increase in the catalytic efficiency. And the gold nanoparticles could also work in coordination with HRP to catalyze DAB deposition to strengthen scattering intensity due to the scattering and mimic enzyme catalysis properties. As a result, the breast cancer sections labeled by the HRP-AbII-Au nanoprobes(Figure 3c) had much stronger scattered light and provided more clearly strctural information of malignant cells in both bright and dark-field than those analyzed by conventional detection method with similar positive degrees(Figure 2), which resulted in an increase in the score of R/S given by DF-Analysis and guaranteed a higher specificity and lower misdiagnosis rate.

Evaluated by TEM (See Supplementary Figure 3ab), UV-vis (See Supplementary Figure 3c) and DLS, the gold nanoparticles (See Supplementary Figure 3a) and the corresponding gold nanoprobes (See Supplementary Figure 3b) had good dispersity. The average TEM diameter and hydrodynamic size of citric acid coated gold nanoparticles were about 14.7 nm and 35.7 nm, respectively. After conjugated with antibodies, the hydrodynamic size increased significantly to 87.0 nm, while TEM size remained unchanged almost. And as measured by BCA kit, the coupling efficiency was 93.1% and the average number of antibodies on a single gold nanoparticle was about 34.

Compared to the dark-field imaging based on the conventional detection method (Figure 4cd), the breast cancer sections and cells labeled by the HRP-AbII-Au nanoprobes had higher-contrast images of bright-field and more strongly scattered light in dark-field (Figure 4ef) at the same time, while the negtive breast tumour sections without label could hardly be seen in the dark-field (Figure 4ab).

Since now we could see that HRP and gold nanoparticle synergistically enhanced dark-field imaging showed obvious advantages against the conventional immunohistochemical method. The synergistically enhanced dark-field imaging provided the distribution information of protein on the surface membranes and clearly structural information of malignant cells (Figure 4g). Compared to conventional method, the synergistically enhanced dark-field images could be quantitatively classified through software analysis, instead of subjective judgment.

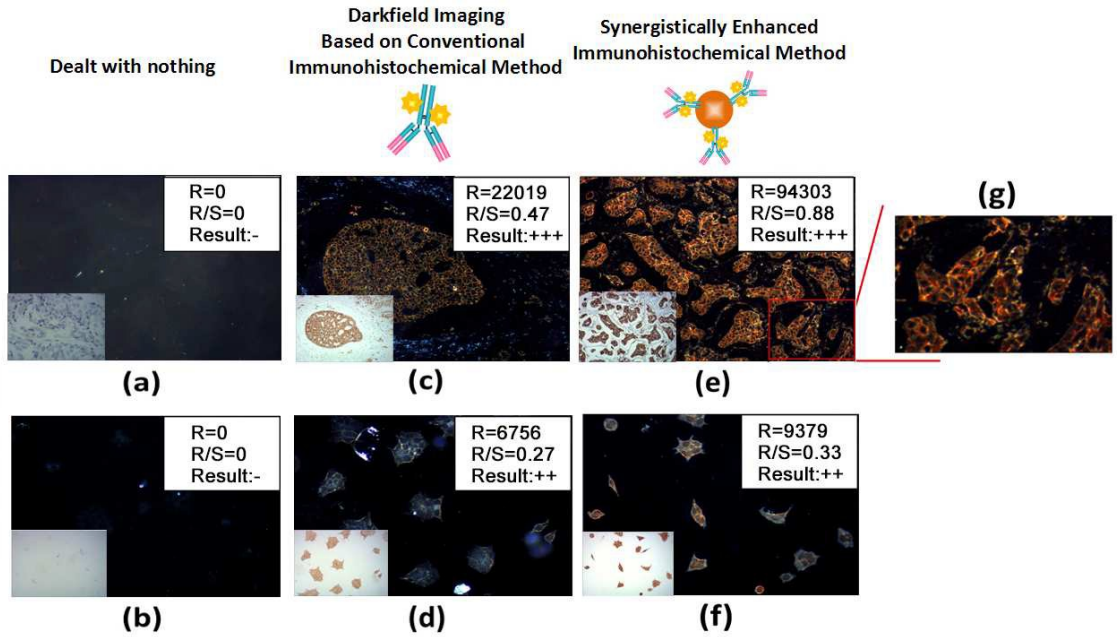

Figure 4. Dark and the corresponding bright field images (100×) of breast tumor sections and BT474 cells with similar positive degree and the analysis results were given by DF-Analysis. Dark and the corresponding bright field images of breast tumor sections (a) and BT474 cells (b) dealt with nothing. Dark and the corresponding bright field images of breast tumor sections (c) and BT474 cells (d) analyzed by conventional immunodetection method. Dark and the corresponding bright field images of breast tumor sections(e)and BT474 cells(f) analyzed by synergistically enhanced immunohistochemical method. Magnification of dark-field image of (g).

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Experimental

Synthesis of gold nanoparticles and nanoprobes.

Citrate reduction method was used for preparing gold nanoparticle colloidal solution. Chloroauric acid(1ml, 0.01g ml-1, Aladdin Industrial Corporation) and trisodium citrate(5ml, 0.01g g ml-1) were mixed in a system with a volume of 100ml and stirred at 100 ℃ for 10 min, and then cooled to room temperature.
The gold nanoprobes were prepared by electrostatic attraction. The pH of 1ml gold nanoparticle colloidal solution was adjusted to approximately 9.5 with 40μl of 0.1M K2CO3 aqueous solution. 15μg Herceptin was mixed with 1ml gold nanoparticle colloidal solution and incubated for 15min. The mixture was then centrifuged to remove unbound antibodies at 2500 rpm for 40 min at 4℃. Deposition was resuspended phosphate buffer solution (PBS) containing 1% BSA to obtain purified Herceptin-Au nanoprobes.
HRP-AbII (Dako), a common HRP labeled secondary antibody for immunohistochemical method, was employed to construct the HRP-AbII-Au nanoprobes. The gold nanoparticles were conjugated with HRP-AbII as the same way we prepared Herceptin-Au nanoprobes with the volume ratio of gold nanoparticle colloidal solution to HRP-AbII of 1ml: 20μl.
The gold nanoparticles as well as the corresponding nanoprobes were evaluated by using transmission electron microscopy (TEM) (JEOL, JEM-2100), ultraviolet visible spectroscopy (Uv-vis) (Shimadzu Scientific Instruments, UV-3600) and dynamic light scattering (DLS) (Brook haven, Zeta Plus). And the coupling efficiency of antibodies on gold nanoparticles was measured by BCA kit (Beyotime Biotechnology Co., Ltd).

Immunohistochemical method for breast tumor sections.

The breast tumor sections were heated at 90℃, then dewaxeded with dimethylbenzene and ethanol of gradient concentration(95%、90%、80%、70%) for 10 min each, and last processed with 3% H2O2 and citrate retrieval solution (Guoyao chemical reagent co., LTD).
114 cases of breast tumor sections with four different expression levels of HER2 were used in the experiment of dark-field imaging based on HRP-catalyzed DAB deposition. The sections were incubated with primary antibodies after the above procedure at 4℃ overnight and washed with PBS-T for 3 times. Then the sections were labeled by the HRP labeled secondary antibodies at 37 ℃ for 30min and stained with DAB for 2min. After washed with PBS-T, the sections were dehydrated with ethanol of gradient concentration(70%、80%、90%、95%) and taken photographs under bright and dark-field microscope(100×).
For the experiment of synergistically enhanced dark-field imaging, the sections were incubated with primary antibodies after the above procedure at 4℃ overnight and washed with PBS-T for 3 times. Then the sections were labeled by the HRP-AbII-Au nanoprobes at 37 ℃ for 30min and stained with DAB for 2min. After washed with PBS-T, the sections were dehydrated with ethanol of gradient concentration(70%、80%、90%、95%) and taken photographs under bright and dark-field microscope(100×).

Immunohistochemical method for BT474 cell smears.

The breast cancer cells of BT474 with HER2 overexpression were used to make smears in the experiment. The smears were processed with 3% H2O2 and Dulbecco's Phosphate Buffered Saline (DPBS).
For the experiment of synergistically enhanced dark-field imaging, the smears were incubated with the primary antibodies at 4℃ overnight and washed with PBS-T for 3 times. Then the smears were incubated with the HRP labeled secondary antibodies at 37 ℃ for 30min and stained with DAB for 2min. After washed with PBS-T, the smears were taken photographs under bright and dark-field microscope(100×).
For the experiment of dark-field imaging based on HRP-catalyzed DAB deposition, the smears were incubated with the primary antibodies at 4℃ overnight and washed with PBS-T for 3 times. Then the smears were labeled by the HRP-AbII-Au nanoprobes at 37 ℃ for 30min and stained with DAB for 2min. After washed with PBS-T, the smears were taken photographs under bright and dark-field microscope(100×).
For the blank controllers, the smears were incubated with PBS-T at 4℃ overnight and washed with PBS-T for 3 times. Then the smears were taken photographs under bright and dark-field microscope(100×).

Conclusions

In summary, we provided a synergistically enhanced dark-field method based on the scattering property of DAB aggregates. Based on the optical property of DAB, we combined conventional immunohistochemistry with dark-field imaging, and developed the quantitative analysis standard and relevant software. As a result, 114 cases of breast tumor sections with different expression levels of HER2 were successfully detected with a sensitivity of

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96.70% and a specificity of 95.65%. In order to obtain a further enlargement of signal, the HRP labeled secondary antibodies conjugated gold nanoparticles were constructed as nanoprobes to load more HRP enzymes, resulting in an enhanced DAB deposition as dark-field label. Besides that, gold nanoparticles could perform synergistically with DAB and HRP due to the scattering and mimic enzyme catalysis properties, which contributed to a significant improvement in both bright and dark-field imaging.

Acknowledgements

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