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Rituximab-Au Nanoprobes for Simultaneous Dark-field Imaging and DAB Staining of CD20 Over-expressed on Raji Cell

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A novel dual-modal cell immunodetection method based on both dark-field imaging and catalysis functions of gold nanoparticles has been established, where the Rituximab-Au conjugates were used as nanoprobes to label and image specifically the CD20 overexpressed on the surface of malignant lymphoma cells of Raji with high affinity.

With the development of nanotechnology, gold nanoparticles exhibit a high potential in tumor inhibition[1], thermotherapy[2], and diagnosis field[3],[4] because of their special optical[5] and electrical properties[6], good stability and surface effect[7], as well as the special size-dependent[8] biological compatibility and limited cytotoxicity[9-10]. El–Sayed et al reported the applications of gold nanorods in both molecular imaging and photothermal cancer therapy[11]. Compared with other shapes, nanorods had longer circulation time in the blood, and higher accumulation in the tumour[12]. In their work, nanorods were modified with antibodies, in order to bind specifically to the surface of malignant cells. As a result of the scattered light from gold nanorods in dark-field, the malignant cells were visualized and diagnosed from the normal ones. Furthermore, the malignant cells can be selectively destroyed using a low-energy harmless near-infrared laser without harming the surrounding nonmalignant cells. In Hu's work[13], specific biomolecules functionalized gold nanorods and silver nanoparticles were used as probes for multiplex dark-field targeted imaging of pancreatic cancer cells due to their widely separated localized plasmon resonance characteristics. Raschke et al[14] also proposed a method for biomolecular recognition using light scattering of a single gold nanoparticle coated with streptavidin, where a spectral shift of the functionalized gold nanoparticle plasmon resonance was induced by specifically combining with biotinlated molecules due to the alteration of dielectric environment around gold nanoparticles.

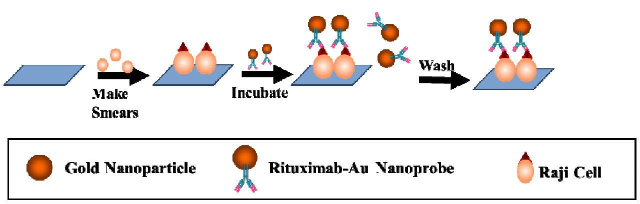
Recently, gold nanoparticles become a mimic enzyme research focus, not only because of their catalytic activities but they also avoid the disadvantages of natural enzymes. He and coworkers[15] demonstrated that gold nanorods coated with a shell composed of Pt nanodots exhibited intrinsic oxidase-like, peroxidase-like and catalase-like activity, which was evaluated using the typical horse radish peroxidase (HRP) substrates like 3,3,5,5-tetramethylbenzidine (TMB) and O-Phenylenediamine (OPD) in the presence of H_2O_2. These new enzyme nanomimetics have competitive advantages in aspects of cost, preparation technology, stability and catalytic activity, and have been applied in bioanalysis instead of HRP, including detection of H_2O_2, glucose, and proteins[15,16].

Fluorescent in situ hybridization (FISH) method[17] and immune histochemical (IHC)[18] technique have become the most common tools in cancer detection. The immunohistochemical technique is simple and low-cost, however, it cannot realize quantitatively detection and relies too heavily on the experiences and subjective judgment to ensure accuracy. For FISH method, which though can realize accurate quantitative detection, the promotion and application was hindered by the high cost. Here a novel dual-modal cell immunodetection method was proposed, which combined both quantitative and low-cost advantages by using gold nanoparticles as probes to dark-field imaging and 3, 3'-diaminobenzidin (DAB) staining of cancer cell membrane molecules. And the two imaging modalities were combined to gain more reliable information in a single picture.The integration of dark-field and bright field imaging mode in the same microscope greatly reduces the cost and improves the speed to get dual-mode image with a simple switch. Comparing with traditional immune cell imaging which uses natural horseradish peroxidase (HRP) to catalyze the chromogenic reaction of substrates, gold nanoparticles as mimetic peroxidase, are lower-cost and easy to prepared and combined to the antibody. At the same time, the dark-field imaging specifically comes from the light scattering of gold nanoparticles, avoiding the negative effect of endogenous enzymes to the catalytic process and the misjudgment resulting from the substrate instability in the bright field imaging. The complementation of the two modes can greatly increases the accuracy.

We employed gold nanoparticles to prepare the Rituximab-Au nanoprobes. The gold nanoparticles with good dispersity as well as the corresponding nanoprobes were evaluated by using transmission electron microscopy (TEM) (Fig. S1, ESI†), UV-vis spectroscopy and dynamic light scattering (DLS) (Fig. S2 and S3, ESI†). The average diameter and hydrodynamic size of bared gold nanoparticles were about 10.0 nm and 16.7 nm, respectively. Rituximab, a commercial therapeutic antibody with CD20 specificity, was used to construct nanoprobes. After conjugating gold nanoparticles with Rituximab antibody, the hydrodynamic size increased significantly to 63.6 nm, while TEM size remained unchanged almost. And as measured by BCA kit, the coupling efficiency of Rituximab on gold nanoparticles was 57.6% and the average number of antibodies on a single nanoparticle was about 7.5.

Malignant lymphoma cell of Raji (with CD20 overexpression) and leukemia cell of K562 (without CD20 overexpression) were used to make smears on microscope slides followed by immobilization with polyformaldehyde, and then incubated with the Rituximab-Au nanoprobes.

We installed an Experimental group and five control groups to study specificity of the detection (Scheme 1 and S1, ESI†). Cells were treated as described below and then observed under both bright-filed and dark-field microscope. The light scattering images were adjusted to a uniform background using Photoshop 8.0.1 (Fig. S4, ESI†). In the experimental group, Raji cells were incubated with the Rituximab-Au nanoprobes at 37℃ for 1 hour and washed by 1% PBS-T three times to remove the redundant nanoprobes (Scheme 1). As a result, the nanoprobes were specifically bound to the overexpressed CD20 on the surface of Raji cells, and the strongly scattering light from the Rituximab-Au nanoprobes on cell membrane was clearly observed in dark-field as expected, with low non-specific binding rate (Fig.1a). The gold nanoparticles without protein coating were also employed as gold nanoparticles group instead of the Rituximab-Au nanoprobes (Scheme S1a, ESI†). In this group, lots of gold nanoparticles were deposited on the surface of cells non-specifically and irregularly since lack of the protection of antibodies and BSA blocking (Fig.1e).



Scheme 1 Labelling process of the Rituximab-Au nanoprobes for CD20 over-expressed Raji cells.

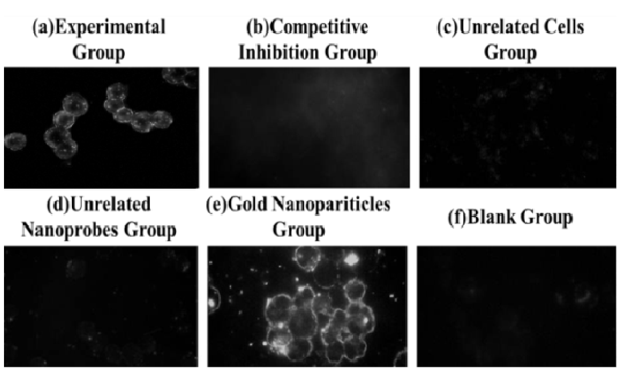


Fig 1 Dark-field images of Raji cells labelled by the Rituximab-Au nanoprobes for the experimental group (a). Other dark-field images for five control groups (b-f) were also described as noted in Figure. All of images were adjusted to a uniform background colour by using an image manipulation software of Photoshop 8.0.1.

In the competitive inhibition group, Raji cells' binding sites was blocked by 100 times amount of Rituximab monoclonal antibodies before incubating with the Rituximab-Au nanoprobes (Scheme S1b, ESI†). For the unrelated cells group and the unrelated nanoprobes group, we changed the cells and the probes to K562 cells (without CD20 overexpress) and human IgG-Au nanoprobes respectively (Scheme S1cd, ESI†). And in the blank group, Raji cells were co-incubated with the buffer of PBS-T instead of gold nanoprobes (Scheme S1e, ESI†). As a result, the four group cells could not be successfully labelled and thus hardly be observed in dark-field (Fig.1bcdf).

Note that, the bright-field images didn't show regularity under different experimental conditions (Fig. S5, ESI†). As fixed with 4% paraformaldehyde to make smears, the Raji cells have been killed already and lost the effects of cellular endocytosis before co-incubated with Rituximab-Au nanoprobes. It is proved that the difference among six groups was due to light-scattering of gold nanoprobes labelled on the surface of the cells rather than the cells themselves or non-specific adsorption. This provided evidence that the scattering intensity reflected the gold nanoparticles bonding situation on the surface of cells.

We installed four inhibition groups by using antibody blocking to indirectly study the correspondence between the amount of gold nanoparticles adsorbed on cells surface and the expression levels of CD20. In the four groups, Raji cells' binding sites (CD20) were blocked by 100(Fig.2a), 50(Fig.2b), 10(Fig.2c) times amount of Rituximab monoclonal antibodies and PBS-T (Fig.2d) before incubating with the Rituximab-Au nanoprobes. With decreasing the amount of Rituximab, the scattering intensity revealed a linear increasing trend due to the increase of effective binding sites of Raji cells. Furthermore, we compared the dark-field (Fig. S6a, ESI†) and the bright field (Fig. S6b, ESI†) image in the same visual field and find that almost 100% cells can be labelld by Rituximab-Au nanoprobes.

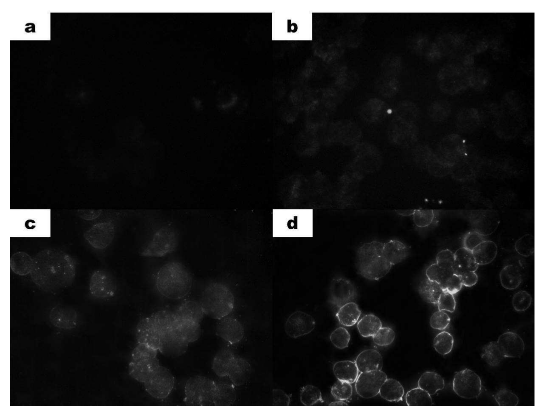


Fig 2 Dark-field image of Raji cells when CD20 binding sites blocked by 100(a), 50(b), 10(c) times and no(d) Rituximab respectively.

Besides that, we used gold nanoparticles as mimetic peroxidase to catalyse colour reaction with DAB as chromogenic substrate in the presence of hydrogen peroxide. Due to the specific binding of the Rituximab-Au nanoprobes on the cells, Raji cells were stained brown by DAB deposition reaction, as observed in bright-field (Fig. 3a). Degree of DAB staining reaction could reflect the amount of the bound nanoprobes on the cells, that is, the degree of the CD20 over-expression. For comparison, cells labelled with the Rituximab-Au nanoprobes but not stained with DAB were clear and colourless in bright-field (Fig. 3b). By comparing the corresponding dark-field images (Fig. 3cd), we found that higher-contrast images of dark-field could also be obtained by DAB deposition reaction, likely due to the enhanced scatter from DAB deposition.

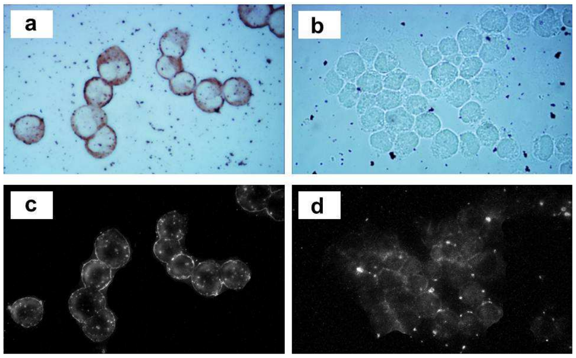


Fig 3 (a) Bright-field image of Raji cells when labelled with the Rituximab-Au nanoprobes which acted as peroxidise to catalyse the colour reaction of DAB. (b) Bright-field image of Raji cells with Rituximab-Au nanoprobe labelling but without DAB staining. (c, d) The dark-field image corresponding to a and b, respectively.

Decreasing non-specific binding plays an important role to improve the specificity and accuracy of tumour detection methods. The Rituximab-Au nanoprobes purified by centrifuging could be completely resuspended in citrate buffer containing 1% BSA or 0.2% PEG20000. BSA was used to block residual binding sites on gold nanoparticle surface, while PEG20000 was used to decrease the non-specific adsorption [19] (Fig. S7af, ESI†). Gold nanoprobe aggregates easily formed during the centrifugal purification and deposited on surface of smears, which enhanced non-specific binding. This could be solved by short-time low-speed centrifugation (Fig. S7fg, ESI†). In addition, dipping slides in PBS-T solution was also useful for decreasing non-specific adsorption (Fig. S7fh, ESI†). With incubating time prolonging, both specific and non-specific binding amount enhanced, so an optimized incubation time of 60 min was selected, which was proved to be able to harvest very weak nonspecific binding (Fig. S7bcf, ESI†). Concentration affected both labeling effects and stability of the nanoprobes. Compared with 57.9 μ g/ml (Fig. S7d, ESI†) and 115.8 μ g/ml (Fig. S7e, ESI†), optimized nanoprobe concentration with 289.5 μ g/ml(OD value of 4.3 at 560nm) (Fig. S7f, ESI†) of gold nanoparticles significantly increased detection sensitivity without leading to the non-specific adsorption and nanoprobe aggregation.

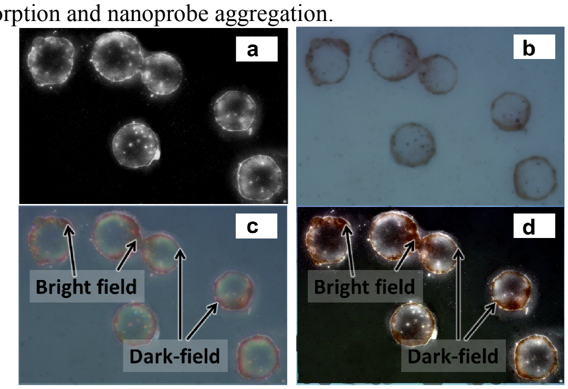


Fig 4 Combination of bright-field image and dark-field image. (a) Dark-field image of Raji cells when labelled with the Rituximab-Au nanoprobes; b. Bright-field image of Raji cells when labelled with the Rituximab-Au nanoprobes and stained with DAB; c. Overlap the two images (a and b) and adjust the opacity; d. Adjust the clarity of the processed images.

In order to gain complementary information in a single picture and achieve more reliable detection results, we overlapped the two images and adjusted the opacity and the clarity of the processed image. In the processed image, we can see Raji cells were stained brown by DAB in bright-field and shown as yellow-white bright circles in dark-field, which could both reflect the amount of the bound nanoprobes on the cells (Fig. 4).

Conclusions

We provide an in vitro detection method using Rituximab-Au nanoprobes as novel contrast agents for cell imaging in both bright-field and dark-field. The Rituximab-Au nanoprobes bind specifically to the surface of Raji cells due to the overexpressed CD20. As a result of the strongly scattered light from gold nanoparticles, the over-expression rate of CD20 is positively correlated to the luminosity of the cells in dark-field. And in bright-field, expression level of CD20 can also be reflected by degree of DAB staining catalyzed by gold nanoparticles with peroxidise-like activity. We combine the two images, in order to gain more reliable information in a single picture. As the scattering intensity can be quantified, there is reason to expect to be able to establish quantificational detection of cell-surface molecule based on gold nanoprobes scattering in dark-field. Relevant software for measuring scattering intensity is under development.

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Notes and references

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† Electronic Supplementary Information (ESI) available: Experimental details, size distribution histograms, electron diffraction patterns, and Raman spectra, see DOI: 10.1039/b000000x/

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