論 文 の 内 容 の 要 旨

論文題目　Development of genetically encoded antibody-FP fusion FRET probes
（細胞で発現可能な蛍光抗体プローブタンパクの開発）

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Biomarkers are measurable substances which could monitor the state of biological processes, pathogenic processes, or pharmacologic responses. Thus, the detection of biomarkers is important to diagnosis, clinical endpoint measurement and disease process measurement. Antibodies are proteins which bind to specific substances, their antigens, through variable regions. Thus, antibodies provide an unlimited natural resource for detecting almost any biomolecules and their modifications as a sensor domain. Immunoassays such as RIA and ELISA are wide-used in biomarker detection with high sensitivity and high specificity. However, these immunoassays usually require several rounds of incubation and washing steps including tedious manipulations, which make them difficult to apply to point of care detection. In addition, conventional immunoassays are not able to trace the dynamic change of protein modifications in living cells or living organisms.

Förster resonance energy transfer (FRET) between GFP variants, are widely used to monitor biological phenomena and biomolecular modification. FRET efficiency is affected by the distance and orientation of fluorescence donor and fluorescence acceptor. Therefore, it could be used to monitor the spatial relationships or conformation changes of interesting molecules. A novel immunoassay based on the interchain interaction of antibody variable region, Open sandwich ELISA, was performed to be a powerful method to quantify low molecular weight antigens. In order to reduce the manipulate of heterogeneous immunoassay, homogeneous immunoassay which using antibody V region as a sensor and a fluorescent protein pair as a reporter was established and was named after open sandwich fluoroimmunoassay (OS-FIA). Such genetically encoded FRET-based probes could be transferred from plasmid DNA clone to the protein probe quickly without need of fluorescence dye labeling. It also processes great potential to be expressed in situ or *in vivo* to monitor biological events without protein

transfection as a long-term non-invasive reporter. In this study, I develop three kinds of FRET probes by fusing antibody variable region or single chain Fv (scFv) to fluorescent protein to detect different biomarkers.

First, a FRET probe for Osteocalcin, also known as bone Gla protein (BGP) as a model for small molecule detection was established. Variation of BGP concentration in blood represents different clinical condition, thus it could be used as a diagnosis of bone metabolism diseases. To image BGP in vitro and also in cellular milieu, a probe pair was constructed by tethering a donor fluorescent protein (FP) to the VH fragment, and an acceptor FP to the VL fragment. The optimized cyan-yellow FP pair for FRET, CyPet-YPet, was used as a donor and an acceptor, respectively. Protein probes were excited at 430 nm in the presence of different concentration of BGP. As the concentration of antigen increased, the overall FRET efficiency was obtained by the fluorescence intensity ratio increased. Titration curve was obtained by plotting the BGP concentration and the fluorescence intensity ratio of Ypet/Cypet. The calculated EC50 was comparable to the IC50 value of KTM-219 Fv obtained by indirect competitive phage ELISA indicates that the BGP peptide-binding activity of BGP probe was not interfered by the incorporation of fluorescence protein in the N-terminal region. This probe is sensitive enough to detect clinically relevant BGP concentration. Therefore this assay might provide a quicker detection of human BGP and its fragments for diagnosis. As a demonstration of live cell imaging, U2OS osteosarcoma cells, which secrete BGP after differentiation into osteoblastoma by vitamin D3 stimulation, were observed. After 36 hrs of induction, the cells manifested stronger FRET efficiency than the non-induced cells. This result suggests that our probe provides an alternative approach to monitor secreted protein from cells directly.

Serum albumin (SA) was used as a target of large molecule protein probe demonstration. The concentration of SA in blood is an indicator to measure the function of liver. Anti- serum albumin scFv obtained from a synthetic phage library and indicated that the interaction between VH and VL fragments of this antibody is fairly strong to form a stable complex even in the absence of antigen. Furthermore, the large molecular dimension of SA may hinder the energy transfer when used for OS-FIA probe. Hence, OS-FIA is not a suitable method for SA detection. To construct a more universal assay to detect large size/dimension of biomarkers, I designed a novel fluoroimmunoassay based on FP-V region fused protein. Two fusion proteins, VH-fused donor and VL-fused acceptor were used, while VH (SA) and VL (SA) with stronger affinity resulting in higher FRET efficiency without antigen. In order to solve this problem, I covalently linked the VH and the VL fragments by introducing two mutations G44C (VH) and Q100C (VL), both in the conserved framework region to make a disulfide-stabilized Fv fragment (dsFv) introducing minimal perturbation in their structure. The VH-fused donor and VL-fused acceptor will form a stable complex and result in high FRET efficiency between the two FPs in the absence of antigen. Once the larger molecule antigen is added, the FRET efficiency will be

decreased, which could be utilized for quantifying antigen. This novel assay using antibody-FP fusion FRET probe FP_2-dsFv is named as “open flower” fluoroimmunoassay (OF-FIA).

When VH-fused donor and VL-fused acceptor complex were stabilized through disulfide bond, extremely high FRET efficiency due to the dimerization of fluorescent donor and acceptor was observed. This effect results in much higher ΔFRET index (13.5 to 3.5) than other FRET probes. FP_2-dsFv probe guarantee that the concentrations of donor and acceptor are the same which reduce the variation form different detections. The effect of human serum albumin (HSA) on overall FRET efficiency was represented by the fluorescence intensity ratio of Ypet/Cypet. The binding of large SA would separate the interacting FP pair apart, thus the energy transfer between the two FPs would be reduced. The calculated IC50 was 32.8 ±1.0 μM, and the limit of detection (LOD) was 0.55 μM. Although the sensitivity of FP_2-dsFv SA probe is modest compared with other scFv-fused FRET probes, the working range is wider. The IC50 for human serum albumin is sufficient to distinguish its normal range (500-800 μM) in serum.

A novel genetically encoded Quench-based antibody probe could be constructed by introducing Y145W/H148V mutations to acceptor fluorescent protein in FP_2-dsFv(SA) probe. The Y145W/H148V Ypet (dark Ypet) received energy form Cypet but emited weak fluorescence as a quencher. Genetically encoded Quenchbody demonstrated antigen-dependent fluorescence enhancement, as observed for the conventional Quenchbodies labeled with organic dye(s).

The third probe is a FRET-based probe for quantifying the histone modification level in living cells. Post-translational histone modifications including acetylation and methylation at the N-terminal tails are the major regulator of gene expression. Abnormal histone modifications cause the repression of tumor suppressor gene or activation of oncogene, thus they are also biomarkers of carcinogenesis. Nowadays, histone modifications are usually detected by immunostaining or activity assay of histone modification enzymes *in vitro*. The conventional assays could not provide enough information about temporal and spatial dynamic changes in living cells and organisms. Two research groups have reported FRET-based probes to monitor the dynamic changes of histone modification in a single cell. The general design of FRET-based probes mentioned above is that fusing a substrate peptide or protein of histone modifying enzymes to modification binding protein as a sensor and FRET fluorescent protein pair in N-terminal and C-terminal ends as a reporter. However, the dissociation constant Kd of FRET-based probes are around 1-200 μM, which means they could barely binding to endogenous histone. In addition, many modification binding proteins are not specific enough, and can bind several modification sites. Taken together, those FRET-based probes reflect the activity of histone modifying and demodifying enzymes, but they are incompetent to monitor the modification of specific site.

A getically encoded histone modification fluorescent probe, modification-specific intracellular antibodies (mintbodies), was recently developed. The probe has succeeded in monitoring histone acetylation level in cultured cells and living organisms by measuring the fluorescence intensity of GFP in the nucleus. In this study, I applied mintbody strategy to construct a FRET-based histone modification sensor. The FRET probe is composed of YFP at the N-terminal of ScFv for acetyl H3K9, and tethered CFP with histone H3 tail at the C-terminus. Once the histone H3 tail is acetylated, it would bind to the single chain Fv to trigger the probe's conformational change. Therefore, the histone acetylation level would be estimated by FRET efficiency.

To verify the validity, probe-transfected U2OS cells were used as a model. The FRET efficiency of transfected cells treated with or without histone deacetylase inhibitior, trichostatin A (TSA), was measured by fluorescence microscopy. The FRET efficiency is lower in cytoplasm than nucleus even without TSA treatment. In nucleus, FRET efficiency increased when the cells were treated with TSA. On the contrary, FRET efficiency was not changed in cytoplasm. The results mentioned above indicates that the intrabody-based FRET probes could measure histone H3K9 acetylationn levels in cultured cells by quantifying FRET efficiency.

In order to elucidate whether our probe is able to detect the acetylation level of endogenous histone or only the activity balance of histone modifying enzyme, H3Lys9 in the C-terminal ends of our probe was mutated into Ala and Gln. K9A mutant peptide could not be acetylated thus we could exclude the effect of acetylation at H3K9 peptide of the probe. K9Q is an acetyl-Lys mimic mutant to compare the basal FRET efficiency. The FRET efficiency of cells transfected with different probe was measured in a time-lapse after challenging with high concentration of TSA. As a result, FRET efficiency and fluorescence intensity in nucleus increased time dependently for all types of probes. It suggests that our probe directly associates with endogenous histone tails rather than binds to its own tail. Wild type probes displayed the highest response to TSA. This might be resulted from the acetylation of H3 peptide of the probe and endogenous histone simultaneously. This probe is the first genetically encoded single chain FRET probe which utilize scFv as a sensing domain and also the first probe that visualize the modification of endogenous protein in single cell level.

In summary, this study demonstrated three types of genetically encoded antibody-based FRET probes which provide universal approaches to detect different kinds of biomarker in homogeneous condition. The amount of analytes could be estimated by FRET efficiency. These probes will have a great potential as a diagnostic reagent and provide a simple approach to monitor cellular differentiations and epigenetic changes at the single cell level.