Abstract

Establishment of competitive FRET methods for the development and application of fluorescent assays

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Introduction: Specific biomolecules must be accurately measured and monitored for basic research and diagnosis. Fluorescent molecules are important tools for designing biosensors. This study establishes basic technologies for designing and applying fluorescent biosensors. Fluorescence energy resonance transfer signals are generated when fluorescent ligands (FLs) bind to the IP_3R ligand-binding domain fused with enhanced cyan fluorescence protein. Fluorescence changes (caused by competition between FL and unlabeled IP_3), can be used to measure IP_3 concentration. This method is called Competitive Fluorescence Ligand Assay for IP_3 (CFLA-IP_3), and this principle can be used to develop sensors to study other molecules. In the present study, I develop two new methods that expand on CFLA: CFLA-biotin, which is based on the biotin–avidin interaction, and Competitive Fluorescence Antigen Assay (CFAA), which is based on interaction between antigens and antibodies. To use CFAA and CFLA-biotin for more practical applications, I developed a sensor carrier using the nonwoven fabric made of the carboxylic acid-modified silica.

Methods:

Construction and expression of LBPs with circularly permuted ECFPs: Ligand-binding proteins (LBPs) were constructed by inserting circularly permuted ECFPs between the second and third α-helices of the LBD. The resulting constructs (cpC-LBPs) were transfected into COS-7 cells for expression by using LipofectAMINE 2000.

Experiments with fluorescent cells and beads: In the CFLA-IP_3 cell assay, cpC-LBP–expressing cells were permeabilized with saponin, and changes in fluorescence were measured after adding a high-affinity fluorescent ligand (F-ADA), a low-affinity fluorescent ligand (F-LL), or both F-LL and IP_3. In the bead-based assay, cytosolic cpC-LBPs were extracted and captured by DYK beads (cpC-LBP beads). Changes in bead fluorescence after addition of F-ADA, F-LL, or both F-LL and IP_3 were monitored with an imaging system. In the CFLA-biotin method, avidin beads were labeled with the amine-reactive blue fluorescent dye Chromis425N (Ch425) and then incubated with FRET acceptor-conjugated biotin (F-biotin) either with or without unlabeled biotin. In the CFAA-DYK method, anti-DYK antibodies on agarose beads were labeled with the thiol-reactive blue fluorescent dye DACM. The fluorescent beads were then incubated with fluorescein isothiocyanate (FITC)-labeled DYK peptides (FT-1) either with or without unlabeled DYK peptide (FT-2). In the CFAA-IP_3R method, Protein G beads were incubated with anti-IP_3R_2 antibody labeled with DACM. These fluorescent beads were then incubated with FITC-labeled antigen peptides (F-CT-18) either with or without unlabeled peptide (CT-18). Bead fluorescence was measured by using an inverted fluorescence microscope with a digital camera.

Experiments with nonwoven fabric: Nonwoven fabric with surface-exposed carboxylic acid moieties (NWF-COOH) was activated by adding *N*-hydroxysuccinimide (NHS) and carbodiimide (EDC), which allowed formation of an NHS ester for efficient conjugation to primary amines in peptides and in biotin-polyethylene glycol-amine. NWF fluorescence was measured with a fluorescence-detecting plate reader.

Results and Discussion:

CFLA experiments using the ligand-binding domain of the IP_3 receptor (CFLA-IP_3): Application of 100 nM F-ADA increased FL fluorescence by ~60% and decreased CFP fluorescence by ~70%, resulting in a decreased fluorescence ratio (CFP/FL) in permeabilized LBP-cpC157–expressing and LBP-cpC173 expressing cells. Application of F-LL (10–300 nM) also reduced the fluorescent ratio in a concentration-dependent manner, whereas subsequent addition of IP_3 restored this ratio. The F-LL EC_{50} value for LBP-cpC157 was 34.7 nM; and for LBP-cpC173, 27.6 nM. IP_3 reduced the FRET signal (generated by 100 nM F-LL) by competing with F-LL. Similar results were obtained with fluorescent beads prepared with cytosolic LBPs. F-ADA-induced changes in the LBP-cpC fluorescence ratios were two-times larger than that of LIBRAvIIS-Vd, indicating that FRET efficiency can be improved by using an appropriate FRET donor.

CFLA experiments using avidin (CFLA-biotin): Since avidin has four biotin-binding sites, I hypothesized that binding of F-biotin to FRET donor-conjugated avidin (F-avidin) would produce a large FRET signal. I tested the use of avidin and biotin in CFLA by comparing several chemical labeling methods. Labeling with 100 μM Ch425N was most effective. Binding of F-biotin to these beads reduced Ch425N fluorescence by 50% and increased F-biotin fluorescence 12 times. An hour incubation with 1 μM F-biotin resulted in an 80% maximal FRET signal, and an overnight incubation with 30 nM F-biotin resulted in a 60% maximal FRET signal. Addition of unlabeled biotin decreased the FRET signal in a concentration-dependent manner. The IC_{50} value of free biotin against FRET with 1μM F-biotin was ~1.5 μM; and against FRET with 30 nM F-biotin, ~50 nM. These results confirm that the sensitivity of the CFLA assay can be controlled by varying the concentration of the fluorescent ligand.

Development of CFAA method: I also developed a version of competitive FRET by using interactions between antigens and antibodies. Binding of FT-1 peptide to DACM-labeled DYK beads reduced the fluorescence ratio (DACM/FT1) to ~22%, and adding unlabeled antigen peptide (FT-2)

restored this ratio to ~77%. Similar results were obtained in experiments with anti-IP_3R_2 antibody and F-CT-18. These results indicate that the CFAA method can be used with antibodies and antigen peptides.

Development of a new sensor carrier using silica nonwoven fabrics: For practical application, I developed a sensor carrier using NWF-COOH. Using confocal laser scanning microscopy, I showed that only a thin surface was found on NWF-COOH fluoresced (< 0.1 μm), whereas a thicker surface was found on DYK beads fluoresced (> 10 μm), and that bead fluorescence extended to the bead interior. The response rate of fluorescent peptide on NWF was 10 times faster than that on DYK beads. Furthermore, I successfully performed CFLA-biotin using an NWF sensor carrier, establishing these reagents for future experiments.

Conclusions: I developed a new version of CFLA, a competitive FRET analysis method, which can be used either with receptor–ligand pairs or the avidin–biotin pair. I also developed CFAA, which can be used with antigens and antibodies. Finally, I developed a method for attaching sensor molecules to NWF-COOH. These technologies improve our ability to measure biomolecular concentrations and interactions by using competitive FRET.