Fluorescence-based applications for Life Science, Biotechnology and Clinical Diagnostics

(An application note)

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Table of contents

1. About this document 3
2. Why use fluorescence in molecular and cellular science? 3
3. What is advantage of time-resolved fluorescence measurements? 3
4. Fluorescence-based applications 4
   4.1. Fluorescence intensity and lifetime-based immunoassays 5
   4.2. Fluorescence polarization-based immunoassays 5
   4.3. FRET-base assays 6
   4.4. Fluorescence correlation spectroscopy (FCS)-based immunoassays 8
   4.5. Intracellular calcium fluxes based assay 10
   4.6. Application of time-resolved fluorescence spectroscopy for studying ligand-induced conformation changes in multi-tryptophan proteins 11
5. References 11

1. About this document

This note reviews fluorescence-based applications employed in life science biotechnology and clinical diagnostics. Most of them can be carried out with use the new generation of fluorescence plate readers, allowing measurements of fluorescence intensity, polarization (anisotropy), and fluorescence resonance energy transfer (FRET) in both the steady state and time-resolved modes.

2. Why use fluorescence in molecular and cellular science?

Fluorescence has found enormously broad scope of different applications in life science and biotechnology, drug design and clinical diagnostics for the last two decades (1). Abundance of these applications is based on the unique scope of fluorescence's characteristics. Emission of fluorescence compounds is characterized by the excitation and emission spectra, the lifetime, and the quantum yield. The fluorescence parameters are sensitive to temperature, polarity and viscosity of solvents, and to various excited state reactions (exciplex formation, electron, proton transfer etc.). In addition, fluorescence emission is polarized and therefore it allows determination of rotational mobility of fluorophores in viscous and liquid solutions. The last but not least important property of fluorescence is based on its quantum character: due to the resonance interaction between electronic systems of two fluorophores, optical excitation can "jump" from one molecule to another by the fluorescence resonance excitation transfer (FRET) mechanism. Since the transfer steeply depends of the distance separating the fluorescence "donor" (D) and "acceptor" (A) molecules, FRET applications allow selective detection of donor-acceptor pairs in solutions.

3. What is advantage of time-resolved fluorescence measurements?

Due to the variety of processes affecting fluorescence probe emission, it is sometimes difficult to attribute observable changes in steady-state fluorescence characteristics to specific mechanisms. For example, changes in steady-state anisotropy could result from changes in the fluorescence lifetime or due to changes in the solvent viscosity. Only time-resolved experiments allow unambiguous interpretation of experimental results. In addition, time-resolved measurements can significantly increase the number of fluorescence-based applications and assays. Recent commercial fluorescence plate readers (e.g. the Fluorescence Lifetime Plate Reader recently launched by Edinburgh

Instruments Ltd.), operating in both the time-resolved and steady state modes, can cover almost all the possible fluorescence applications in life science, biotechnology, drug design and clinical diagnostics.

4. Fluorescence-based applications

One of the most common fluorescence-based applications is to detect and quantitate various ligands such as cytokines, growth factors, and hormones in biological samples. The method is based on interactions of ligands (antigens) with specific antibodies or receptors. Due to the high specificity and affinity of antigen-antibody interactions, these assays can detect picomolar concentrations of ligands. To utilize this assay, one antibody (the "capture" antibody) is usually bound to a solid phase typically attached to the bottom of a plate well. Antigen is then added and allowed to complex with the bound antibody. Unbound products are then removed with a wash, and a labeled second antibody (the "detection" antibody) is allowed to bind to the antigen, thus completing the "sandwich". Monoclonal antibody combinations must be qualified as "matched pairs", meaning that they can recognize separate epitopes (sites) on the antigen so they do not hinder each other's binding. Chromogenic substrate changes color when cleaved by the enzyme attached to the second antibody. A major disadvantage of this assay is its "inhomogeneous" format, i.e. the need to perform the several-step protocol with multiple washes after each step, as well as to perform the final development step. All these significantly elongate the assay and require either a long "bench work" or using expensive instruments to carry out the assay automatically. Technological needs demand development of assays in the homogeneous format, when all steps are combined into one. Several homogeneous fluorescence-based methods will be discussed below.

4.1. Fluorescence intensity and lifetime-based immunoassays

Changes in fluorescence intensity of fluorescently labeled ligands upon binding to receptors can be used for development of homogeneous immunoassays. The changes in fluorescence intensity can result from alterations in the fluorescence lifetime (2). For example, fluorescence intensity of insulin-conjugated fluorescein exhibits 53% increase upon the ligand binding to a specific antibody (3). However, fluorescence intensity can be also changed due to time-resolved spectral shift or changes in quantum yield. Measurements of each of the above parameters can be employed in homogeneous assays, however the lifetime measurements, provide the simplest and the most accurate method for determination of ligand concentrations due to the "self-referenced" character of this parameter: Fluorescence lifetime does not depend on intensity of the excitation or fluorophore concentration (upon employed for lifetime measurements experimental conditions), and therefore lifetime changes can be related to the bound (unbound) fraction concentration.

4.2. Fluorescence polarization-based immunoassays

A significantly more universal assay, operating in a homogeneous format is based on fluorescence polarization measurements (4-6). The assay is broadly used for determination of concentrations of relatively small ligands. The polarization assay uses changes in the rotational mobility of a fluorescently labeled ligand (tracer) upon binding to a specific antibody. Fluorescence anisotropy of a mixture of bound (b) and unbound (u) ligand is given by the following equation:

$$r = r_o \left[\frac{\alpha}{1+\frac{\tau^b{}_f}{\tau^b{}_D}} + \frac{1-\alpha}{1+\frac{\tau^u{}_f}{\tau^u{}_D}} \right], \quad (1)$$

where τ_f and τ_D are fluorescence lifetime and rotational diffusion time constants; r_0 is the limiting anisotropy value and α is the fraction of the bound ligand. This assay is performed in a completion format: a tracer of known concentration and affinity and an unlabeled ligand compete for binding to the same specific monoclonal antibody. Unlabeled ligand displaces the bound labeled ligand and increases the concentration

of the unbound labeled fraction. The method's accuracy is determined by the ratio of the fluorescence lifetime and the diffusion time constants (Eq. 1). Typical lifetime of broadly used for the assay dyes is about 5 ns. Rotation correlation times of 10 and 100 kD proteins are 12 ns and 30 ns correspondently. Eq. 1 yields the maximal anisotropy changes of 13% for 10 kD and 3% for 100 kD proteins. The assay requires very accurate measurements of fluorescence polarization (anisotropy) due to the relatively modest dynamic range. This method has found many applications in clinical diagnostics for detection of drugs, metal ions etc. in biological samples (7-9).

4.3. FRET-base assays

FRET-based format provides an excellent possibility for design of homogeneous assays with a large dynamic range. (10). In order to optimize the method for maximal performance the following criteria for selection for D and A molecules should be taken into consideration. First, the chromophores should have high extinction coefficients and quantum yields and possess of significant photostability. Best D-A pairs should also have high FRET efficiency and emit in the red or near infrared spectral regions where photomultipliers are sensitive but auto-fluorescence of biological samples is relatively low. Several FRET formats can be considered. One possibility is to use a "matched" antibodies pairs. One antibody (Ab1) is labeled with D and the other one (Ab2) with A. The FRET signal appears only when the antibodies and the antigen (Ag) produce the "sandwich" (11).

D-Ab1 + Ag + Ab2- A $\xrightarrow{f}$ **D- Ab1-Ag -Ab2-D**

(2)

Application of labeled with D and A antibody pairs allows assessment of antigens in direct (not competition) format. A special care should be paid for optimization of the capture and detection antibodies concentrations. They should be chosen slightly higher than the expected concentration of the antigen in order to separate the FRET signal from fluorescence of the unbound antibody. One can also reduce the impact of the "directly" excited acceptor excitation by appropriate selection the excitation and emission wavelengths. Monitoring of the donor and acceptor fluorescence in the time-resolved format and following exponential decomposition of the fluorescence kinetics provide an additional possibility for separation of the FRET signal from emission of

unbound antibodies, directly excited acceptor's fluorescence, auto-fluorescence and scattering.

Another possibility to design a FRET-based assay is to directly label an antigen with A and a specific antibody with D.

D-Ab + Ag-A $\rightleftharpoons$ **D-Ab-Ag-A**

(3)

This method can be used only in the competition format but it requires only one specific antibody. The method has found many different applications, e.g. detection of histamine release (12), an important messenger in allergic reactions. An assay is based on the competition between sample histamine and allophycocyanine-labeled histamine for binding to a Europium cryptate-labeled antibody. Due to its high sensitivity (2 to 400 nM), the assay can be employed for screening of histamine inhibitors in drug design or for allergen identification in clinical diagnostics.

The FRET-based method can be also employed for studying protein-ligand interactions without using specific labeled antibodies. We employed this method for studying interactions of antigenic peptides with Class I MHC molecules, which play an important role in functioning of the immune system (13-15). The assay takes advantage of a large number of tryptophan residues in vicinity of the peptide-binding site of the MHC molecule. These intrinsic tryptophans were used as fluorescence donors. In order to monitor the binding reaction time-course dansyl group was covalently bound to an antigenic peptide. Dansyl was chosen due to the following reasons: 1) its emission spectrum shifts from 550 to 480 nm and quantum yield significantly increases upon peptide binding to MHC; 2) a good overlap of dansyl's absorption with tryprophan's emission provides efficient energy transfer conditions. All these allow monitoring of peptide – MHC complex formation in real time when concentration of the unbound peptide is 10 to 50-fold higher than concentration of the MHC molecule. This method provides a basis for screening of large peptide libraries. We suggest the following protocol: Biotinylated MHC complexes should be attached to a streptravidin-coated plate. Then known concentrations of the dansylated peptide and a peptide of interest are added to each well. Binding of the dansylated peptide to MHC is monitored at 480 nm upon 290 nm excitation. Affinities of the competing peptides can be calculated from the known affinity of the dansylated peptide and final

concentrations of the dansylated peptide-MHC complexes. Alternatively, the immobilized on the plate empty MHC molecules can be "pre-loaded" with different peptides of interest and then high concentration of dansylated peptide (10 - 50-fold higher than concentration of MHC) is added to each of the well in order to monitor dissociation time-courses of the "pre-loaded" peptides from MHC. Peptide affinities can be calculated from the peptide dissociation rates. A possible industrial application of the FRET-based screening assay has been discussed with Beckman-Coulter, the biggest supplier of peptide-MHC monomers and tetramers on the market.

4.4. Fluorescence correlation spectroscopy (FCS)-based immunoassays

Although fluorescence fluctuations cannot be measured by the available at the present time plate readers, it is worth to mention this format due to its extremely high potential (16). The method's idea is illustrated in Fig. 1. Laser beam illuminates a small (0.2 fl) volume in a sample containing fluorescently labeled particles. The photo-detector (DET) measures a fluorescence intensity signal from the illuminated volume. The fluorescence intensity is fluctuating due to Brownian diffusion and a small number of particles in the illuminated volume at picomolar concentrations. An example of fluorescence fluctuations is shown in panel A. Panel B shows a fluorescence correlation function that is calculated from the fluorescence fluctuations. If the sample contains particles of two different sizes, the fluorescence correlation is given by the following equation:

$$G(t) = 1 + \frac{1}{N}\left[(1-Y)g\left(\frac{1}{1+\frac{\tau}{\tau_{D1}}}\right)\left(\frac{1}{1+\frac{\tau}{S^2\tau_{D_1}}}\right)^{1/2} + Yg\left(\frac{1}{1+\frac{\tau}{\tau_{D2}}}\right)g\left(\frac{1}{1+\frac{\tau}{S^2\tau_{D2}}}\right)^{1/2}\right],$$

(4)

where N is the number of fluorescent particles, S is a structural parameter of the experimental setup, τ_{D1} and τ_{D2} are a diffusion times of the particles, and Y is the fraction of the particles with the τ_{D2} diffusion time. By fitting the experimental correlation function to this model, one can get the particles concentrations and their diffusion times.

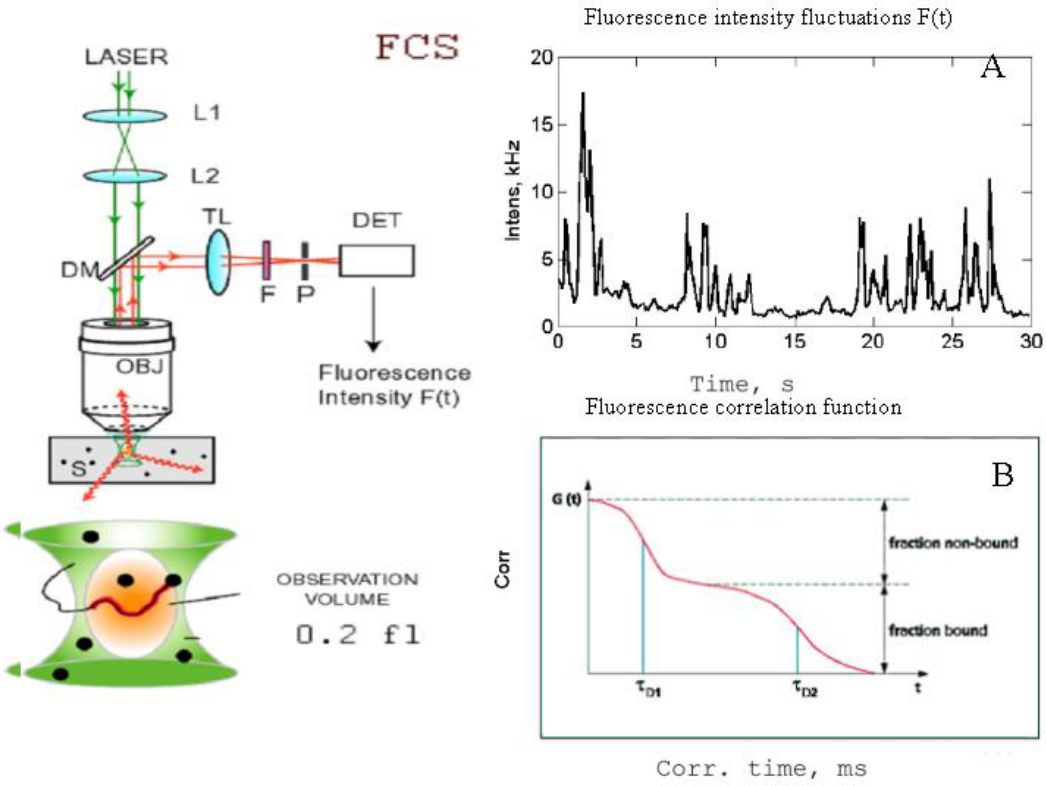

Fig. 1. FCS method.

This method allows several simple protocols for determination the antigen concentration. One of the protocols, based on a "matched" pair of antigen-specific antibodies is illustrated in Fig. 2

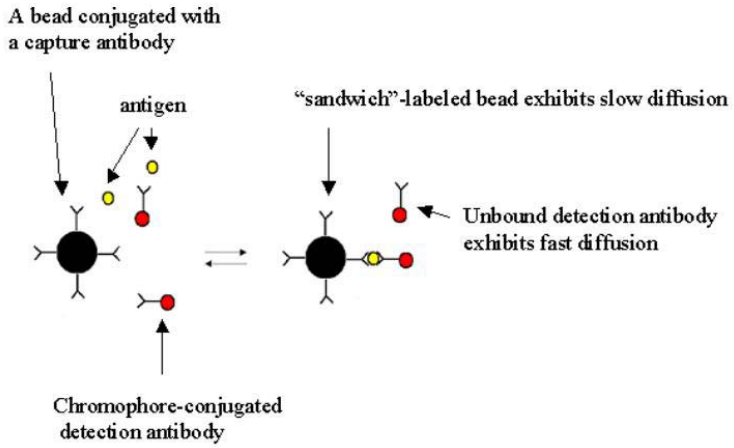

Fig. 2. FCS-based homogeneous immunoassay

A capture antibody is attached to small beads. The bead's size is chosen such that its diffusion coefficient is significantly slower that that of the "free" antibody. The detection antibody is labeled by a suitable fluorophore. Upon the "sandwich" formation, the bead becomes fluorescently labeled. If the concentration of the detection antibody is about equal to the antigen concentration, the fluorescence fluctuations of the labeled beads can be easily separated from the fluctuations produced by the unbound labeled antibody. The antibody-conjugated bead concentration, however, should be used significantly higher than the antigen concentration in order to accelerate binding and ensure production of only one "sandwich" on each bead. This will provide a simple relationship between the beads and antigen concentrations. An important advantage of the FCS-based assay is the possibility to work with very small volumes (40 μl) of biological samples and thereby minimize the amount of relatively expensive antibodies. In addition, the FCS format allows simultaneous analysis of several antigens by either using different chromophores for labeling of each detecting antibody or using beads of different size for each capture antibody. Due to the homogeneous formats, the assay can be employed for monitoring of cytokines secretion by adhesive or suspension cells in response to biological stimuli and therefore can be successfully used for high throughput screening in drug design.

4.5. Intracellular calcium fluxes based assay

The measurement of intracellular calcium response in living mammalian cells is a popular functional assay for identification of agonists and antagonists to receptors of pharmacological interest (17). Development of new calcium-sensitive probes has provided a basis for applications of this method in high throughput screening (HTS). The principle of calcium detection is based of change in quantum yield or spectral properties of fluorescent probes in the calcium bound and free forms. Ratiometric dyes, exhibiting shift in either fluorescence or emission spectra, can be used for quantitative calcium determination. Correlating magnitudes of the calcium response with concentrations of test compounds, pharmacological responses of cells can be studied.

4.6. Application of time-resolved fluorescence spectroscopy for studying ligand-induced conformation changes in multi-tryptophan proteins

The large number of tryptophans in MHC molecules facilitates detection of conformational alterations in MHC protein upon peptide binding (18). Due to the high tryptophan "density", a homo-energy transfer among the tryptophans takes place. The energy transfer induces the tryptophan emission spectral shift towards the red spectral region on the pico- and nano-second time-domains. Since the rate of this shift depends on the distances between the tryptophans within the protein, it can be taken as a characteristic of the protein structure. By comparing the rates of the spectral shift between loaded and empty MHC proteins, structural changes in the MHC molecule induced by peptide binding can be detected. This method is of general significance and can be employed for studying ligand-induced conformational changes in many different multi-tryptophan proteins.

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