Chapter 7

Fluorescence-Based Assays

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Summary

Fluorescence-based assays are widely used in high-throughput screening due to their high sensitivity, diverse selection of fluorophores, ease of operation, and various readout modes. As a result, fluorescence-based assays have been applied to monitor a broad range of activities in life-science research such as molecular dynamics and interactions, enzymatic activities, signal transduction, cell health, and distribution of molecules, organelles, or cells. This chapter describes two fluorescence-based techniques: total intensity measurement as an indication of cell viability, and fluorescence resonance energy transfer as an indication of protein folding and interactions, to illustrate in detail applications suitable for cell-based high-throughput screening in plate-reader and automated microscope-based formats, respectively.

Key words: Automated microscope, Calcein, Cell-based, Fluorescence, Fluorescence resonance energy transfer, High-content image-based screen, High-throughput screen, Nuclear translocation.

1. Introduction

Fluorescence-based detection is arguably the most dominant applied detection method in life-science research, particularly in applications that involve high-throughput screens (HTS). This widespread use is largely due to very high sensitivity, diverse selection of fluorophores that excite and emit across a broad spectrum of wavelengths, and variety of types of readouts based on different environment-sensitive fluorescence properties. These unique characteristics of fluorescence-based detection techniques allow miniaturization, flexibility in assay design, ease of operation, and simultaneous monitoring of multiple events, thus making fluorescence-based assays quite amenable to HTS.

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Fluorescence-based assays can be generally divided into two classes. The first class encompasses techniques that macroscopically detect the total fluorescence intensity, fluorescence polarization, fluorescence resonance energy transfer (FRET), fluorescence lifetime, time-resolved fluorescence, and combinations of these techniques, such as time-resolved fluorescence polarization. The second class of fluorescence-based assays detects fluorescence from single fluorescent molecules, such as fluorescence correlation spectroscopy and fluorescence intensity distribution analysis. These fluorescence techniques have been used to monitor an enormous collection of biological processes, such as macromolecule–macromolecule interactions, macromolecule–small molecule interactions, enzymatic activities, signal transduction, cell health, and states and locations of molecules, organelles, or cells [for general information on fluorescence and its applications, *see (1–3)]*. Given the tremendous scope of fluorescence-based applications, as well as the emphasis of this textbook on cell-based high-throughput assays, this chapter will not endeavor to cover all these techniques and applications. Instead, it will focus on two commonly used techniques: a total fluorescence intensity measurement using a fluorescent dye, calcein, as an indicator for cell viability, and FRET measurement in an image-based format using cyan-fluorescent protein (CFP) and yellow-fluorescent protein (YFP) as an indicator for intramolecular folding and intermolecular association of proteins. The following protocols apply to batch processing with individual instruments, with emphasis on the conditions and procedures that impact on assay biology, as opposed to certain other important operational aspects of HTS, such as compound management and informatics.

2. Materials

2.1. Calcein-AM Viability/Cytotoxicity Assay

2.1.1. Assay Background

Calcein-AM is the acetoxymethyl ester (AM) derivative of calcein. There are two significant differences between calcein-AM and calcein. Calcein-AM is colorless and nonfluorescent until hydrolyzed into calcein. Calcein-AM is cell membrane-permeable and can readily enter cells by diffusion. In contrast, calcein is fluorescent and, owing to its six negative charges and two positive charges at neutral pH, is cell membrane-impermeable *(4)*. Therefore, once calcein-AM enters cells with an intact membrane, the AM-ester moiety will be hydrolyzed by nonspecific esterases in live cells and the resulting product, calcein, will be trapped inside such cells, generating fluorescence upon excitation. Calcein's superior cellular retention over other popular fluorophores, such as fluorescein and BCECF, bright fluorescence insensitive to physiological pH,

and lack of interference with several cellular processes make it, in its AM-ester form, a widely used fluorescent probe to mark live cells with an intact cytoplasmic membrane *(4)*. Specifically, calcein-AM has been used to measure cell viability and cytotoxicity (*see* **Note 1**), membrane permeability, gap-junction formation, cell adhesion, and multidrug resistance.

2.1.2. Consumables

1. *Cell culture media*. The following protocol applies to virtually all adherent cell types. Because of this, no cell lines and growth conditions are specified here, but most cells grow with their base media supplemented with 5–10% fetal bovine serum (FBS; HyClone, Ogden, UT). Inclusion of antibiotics in media is encouraged, as long as the antibiotics of choice (e.g., penicillin and streptomycin; Invitrogen, Carlsbad, CA) are compatible with the cell line-specific culture conditions. This protocol should apply independently of common medium types and FBS percentages.
2. Trypsin-EDTA (Invitrogen).
3. Phosphate-buffered saline (PBS; Invitrogen).
4. Calcein-AM (Invitrogen).
5. 384-Well black, clear-bottomed plates (*see* **Note 2**).

2.1.3. Instrumentation

1. Fluorescent plate reader with top-reading capability. Many brand names will work; however, those with stackers, such as the EnVision (PerkinElmer, Waltham, MA) and Synergy (BioTek, Winooski, VT), provide much better throughput.
2. Bulk dispenser with plate stackers, such as μFill (BioTek), WellMate (Matrix Technologies, Hudson, NH), and the Multidrop (Thermo Scientific, Waltham, MA).
3. Plate washer with plate stackers, such as ELx 405 (BioTek).
4. Compound-transfer instrument that is able to deliver nanoliter (nL) range of com1pounds, such as the CyBi-Well (CyBio, Woburn, MA) equipped with a 384-pin head.
5. Tissue culture incubators.

2.2. CFP-YFP-Based FRET Measurement in Conjunction with Fluorescence-Based Nuclear Translocation Monitoring in an Image-Based, High-Content Assay

2.2.1 Assay Background

FRET is a distance-dependent energy transfer mechanism, by which the excited state of the donor fluorescent molecule is transferred to a second, acceptor fluorescent molecule without fluorescence radiation from the donor *(1)*. FRET requires that the emission spectrum of the donor fluorophore overlaps with the excitation spectrum of the acceptor fluorophore, and that these two fluorophores are in close proximity (10–100 Å) *(1)*. The distance-dependent property of FRET is employed extensively in bioscience to monitor complex formation or dissociation, or any activity that may cause complex formation or dissociation, such as ligand binding-induced conformational changes *(4)*. CFP

(as donor) and YFP (as acceptor) constitute a pair of fluorophores that have been widely used as FRET-generating partners (*see* **Note 3**). This is because the emission spectrum of CFP overlaps with the excitation spectrum of YFP. Therefore, CFP-to-YFP FRET takes place when respective CFP- and YFP-tagged macromolecules, such as proteins, peptides, or DNA sequences, are brought sufficiently close to each other through a biological mechanism, such as intramolecular folding, protein–protein, protein–DNA, and DNA–DNA interactions, or ligand binding.

The following is an outline of an image-based application of CFP-YFP FRET to identify small-molecule modulators of intramolecular folding and/or dimerization of androgen receptor (AR) using a CFP-AR-YFP fusion protein (C-AR-Y) expressed in HEK293 cells. The application uses an automated epifluorescence microscope that is suitable for high-content screening. In addition to intramolecular folding/dimerization of AR indicated by increased FRET signals upon stimulation of dihydrotestosterone (DHT, ligand for AR), the high-content format of this image-based application allows the simultaneous monitoring of another critical feature of AR function, AR nuclear translocation, by tracking cellular location of each fluorescence tag (*see* **Note 4**).

2.2.2. Consumables

1. Cell lines and cell culture conditions.
   a. HEK293 cells (*see* **Note 5**).
   b. An HEK293 cell line stably transfected with a constitutively expressing C-AR-Y construct (*see* **Note 5**). This line is hereafter referred to as C-AR-Y/HEK.
   c. *Culture media*. For HEK293: DMEM (Invitrogen) supplemented with 5% FBS and 1× penicillin/streptomycin mixture. For C-AR-Y/HEK: the same medium for HEK293, further supplemented with 5 μg/mL hygromycin (Invitrogen).
2. Trypsin-EDTA (Invitrogen).
3. DHT (Sigma-Aldrich, St. Louis, MO).
4. Formaldehyde, 37% (Sigma-Aldrich).
5. Hoechst 33342 (10 mg/mL, Invitrogen).
6. Phosphate-buffered saline.
7. 384-well black, clear-bottomed plates (Corning Costar, Lowell, MA; *see* **Note 6**).
8. Opaque plate seals (PerkinElmer).
9. Fix solution: 4% formaldehyde and 0.5 mg/mL Hoechst in PBS. Keep at 37°C before use.
10. 1% Tergazyme (Alconox, White Plains, NY).

2.2.3. Instrumentation

1. Automated microscope for high-content imaging (ImageXpress Micro; Molecular Devices, Sunnyvale, CA; *see* **Note 7**).
2. Bulk-liquid dispenser with plate stackers.
3. Plate washer with plate stackers.
4. Compound-transfer instrument that is able to deliver a nanoliter (nL) range of compounds, such as the CyBi-Well (CyBio) equipped with a 384-pin head.
5. Tissue culture incubators.

3. Methods

3.1. Calcein-AM Viability/Cytotoxicity Assay

1. Cell culture and dispensing cells onto 384-well plates.
   a. Grow adherent cells to near-confluence and avoid overcrowding. Most types of cells grow at 37°C and 5% CO_2.
   b. Perform a trypsin–EDTA digest to remove cells from the flask surface and into suspension. It is critical to disrupt clumps of cells by pipetting the suspension up and down. A well-dispersed suspension allows accurate cell counting, and reduces chances of uneven distribution of cells and clogging of the fine nozzles of the bulk dispenser.
   c. Seed 1,000–5,000 cells per well in 50 µL of medium using a bulk dispenser. To maintain a sterile environment, the dispenser is best placed in a biosafety cabinet with laminar airflow, or in a benchtop enclosure with reversed and filtered airflow. Plates will be delidded in such a clean environment, stacked for bulk dispensing, and relidded. Tubing and other plumbing components can be sterilized with 70% ethanol. To ensure that cells reach the bottom of the wells, the rate of dispensing needs to be adjusted so that it is fast enough to allow the solution to reach bottom of wells without creating air plugs, but gentle enough not to damage the cells. If air plugs prevent cells from settling, centrifugation of the plates usually removes the air plugs and allows the cells to settle properly onto the bottom of the wells.
   d. Incubate seeded plates overnight in tissue-culture incubators.
2. Pin-transfer 100 nL of compound per well. Depending on the stock concentration and experimental design, the final concentration of the compounds can range from approximately 5 to 30 µM (*see* **Notes 8** and **9**).

3. Incubate the compound-treated cells in tissue-culture incubators for the period of time designed to address specific experimental questions (e.g., 24–72 h).
4. Aspirate the compound-containing medium using a plate washer and replace with 50 µL per well of PBS using a bulk dispenser (*see* **Note 10**). Care is needed after cells are incubated with compounds, because cells in certain wells may become fragile due to compound treatment. Aspiration speed and plate-washer pin height (distance between the tips of the pins and the bottom of the wells) both need to be adjusted so that minimal residual liquid will remain, and so that as little cell loss as possible will occur. Again, the dispensing rate on the dispenser needs to be gentle enough not to dislodge the cells.
5. Aspirate the PBS wash with the plate washer and add 30 µL per well of 1-µM calcein-AM in PBS with the bulk dispenser. To make this 1-µM calcein-AM solution, the stock solution of calcein-AM (1 mg/mL, or 1 mM, in DMSO) is diluted 1:1,000 into PBS. Incubate plates for 1 h at room temperature to load calcein-AM into cells (*see* **Note 11**).
6. Wash cells three times with 50 µL/well of PBS, using plate washer to aspirate and bulk dispenser to dispense (*see* **Note 12**).
7. Measure fluorescence intensity on a fluorescence plate reader. Calcein's excitation (ex) and emission (em) maxima are approximately 494/517 nm. However, the green-fluorescein channel (approximately ex/em 485/530 nm) can be used without compromising the signal intensity, if investigators are limited by the predetermined physical properties of the filter sets available to their fluorescence plate readers. When using a filter-based fluorescence plate reader, it is also important to make sure that the proper dichroic mirror is used. A good dichroic mirror eliminates excess excitation light that could interfere with the emission light when no dichroic mirror is used. Also make sure to follow the fluorescence plate reader's steps to optimize plate dimensions, detector height, detector gain, and so on, if such options are offered.

3.2. CFP-YFP-Based FRET Measurement in Conjunction with Fluorescence-Based Nuclear Translocation Monitoring in an Image-Based, High-Content Assay

1. *Cell culture*. Grow sufficient number of C-AR-Y/HEK and HEK293 cells to approach confluence in tissue culture incubators at 37°C and 5% CO2 to be used on "day 1" of the screen. Use trypsin–EDTA to remove the adherent cells and resuspend cells in their respective media.
2. Dispense DHT-containing or DHT-free media onto screening plates. Label the assay plates and a control plate in advance (e.g. using prebarcoded plates). One the screening day, dispense

20 μL of 25-nM DHT in medium to wells on screen plates using a bulk dispenser. Dispense to wells on the control plate as follows: 20 μL respective growth media to columns 1–6 (C-AR-Y/HEK) and columns 13–18 (HEK293) and 20 μL of 25 nM respective growth media containing DHT to columns 7–12 (C-AR-Y/HEK) and columns 19–24 (HEK293).

3. Pin-transfer 100 nL of compounds per well into screen plates. Pin-transfer DMSO into the control plate.
4. Dispense cells into screen plates and the control plate. Using a bulk dispenser, seed C-AR-Y/HEK cells at approximately 10,000 cells per well in 30 μL of medium in screening plates to which compounds have been added. Seed HEK293 and C-AR-Y cells at approximately 10,000 cells per well in 30 μL of respective media into the control plate as follows: C-AR-Y/HEK in columns 1–12 and HEK293 in columns 13–24. Incubate all plates for 24 h at 37°C and 5% CO_2 (*see* **Note 13**).
5. Preparing cells for imaging.
   a. Aspirate the media from the wells in the screening plates using a plate washer.
   b. Fix cells by adding 50 μL of fix solution to each well, and incubate at room temperature for 30 min.
   c. Aspirate the fix solution and wash twice with 50 μL of PBS. Seal the plates with opaque plate seals.
6. Acquire images using the ImageXpress Micro. Four fluorescence channels are needed for a complete set of images. The specific filter sets used for these channels are as follows (excitation/lower transmitted limit of dichroic mirror/emission wavelengths, all in nm): Hoechst (387/415/447), CFP (438/467/483), YFP (500/528/542), and FRET (438/467/542). The Hoechst channel identifies the nucleus, while the other three channels identify the C-AR-Y fusion protein. Both laser- and image-based focusing mechanisms are used to ensure the best focus for each image. The camera is set to 2 × 2 binning of pixels. With a 20× objective, approximately 200 cells can be imaged per field; additional cells can be imaged by acquiring additional fields. Avoid exposure saturation. It takes approximately 45–55 min to image a 384-well plate.
7. Images and data analysis:
   a. *Fluorescence intensity and FRET*. The acquired images are analyzed with MetaExpress (Molecular Devices Corp.). The *Cell Scoring* module is used to generate fluorescence intensity values for CFP, YFP, and FRET channels in both nuclear and cytoplasmic areas in a given well. These per-well values are averaged from all cells sampled in these wells with units of fluorescence units/μm^2.

The HEK293 cells on the control plates provide information regarding background fluorescence intensity for DHT-treated or untreated cells; such background values need to be subtracted from the raw fluorescence reading of C-AR-Y/HEK cells. FRET is usually expressed as a ratio of the signal of the FRET channel over the donor (CFP) channel (*see* **Note 14**).

b. *Nuclear translocation*. The YFP channel is used to track C-AR-Y fusion protein, and the *Translocation* module is used to generate information of percent of cells with nuclear C-AR-Y fusion protein. For the specific C-AR-Y/HEK cell line used, approximately 70–90% of cells undergo nuclear translocation in the presence of DHT, as opposed to under 10% of cells in the absence of DHT.

4. Notes

1. As a cytotoxicity indicator, calcein-AM-based assays provide only live/dead information. They do not suggest specific mechanisms by which cells die or by which cell membranes are compromised.
2. Clear-bottomed plates are ideal to examine cells visually under a microscope. If there is no intention of examining the cells during the assay, opaque-bottomed black plates can also be used. Many vendors' plates are good for this application, such as Nunc and Corning brands.
3. Until recently, CFP and YFP have been the classic and most popular FRET partners for protein applications. In the last few years, there have been reports of alternative FRET partners using DsRed in combination with CFP and GFP, respectively, that demonstrate certain advantages over the CFP-YFP pair *(5, 6)*.
4. AR belongs to the nuclear receptor superfamily and mediates androgenic functions. Upon binding to its ligands (e.g., DHT) in the cytoplasm, AR undergoes a conformational change that allows its N-terminal domain to bind to its C-terminal domain *(7–9)*, followed by homodimerization, entry into the nucleus, and transcriptional activation of target genes *(9, 10)*. Significantly increased FRET signal is generated in the presence of DHT, as opposed to in the absence of DHT, in cells expressing C-AR-Y *(9)*.
5. The HEK293 and C-AR-Y/HEK lines used by the author were kindly provided by Dr. M.I. Diamond, University of California,

San Francisco. The HEK293 line is also available from American Type Culture Collection (ATCC, Manassas, VA).

6. Some brands of plates are better than others for image-based assays, presumably due to the variation in the flatness and thickness of the plastic plate bottom. Investigators are encouraged to test out a few brands with their instrumentation, and to select those that perform the best in their own hands.
7. This protocol should accommodate automated microscopes from other vendors, although different brands have different acquisition settings and use different image analysis software.
8. To reduce protein binding by compounds, growth medium with a high FBS percentage can be changed to one with a lower FBS percentage, such as 0.5% FBS, during compound exposure. This modification will introduce an extra medium-change step, and cells need to be tested to ensure that they will survive such low FBS conditions for the intended duration of compound exposure.
9. Compounds from stock plates are typically in 100% DMSO. During the assay development stage, cells need to be tested to determine if they can tolerate the final DMSO concentration. If cells are found to be sensitive to the intended final DMSO concentration, the volume of cell-culture medium into which the compounds are pinned can be adjusted to reduce the final DMSO concentration to some degree, but this alteration needs to be balanced against the subsequent decrease in compound screening concentrations.
10. It is important to make sure that the plate washer aspirates all wells uniformly in a plate. Occasionally, a few wells in a plate may be missed due to suboptimal vacuum pressure or clogging of individual nozzles. When clogging happens, run maintenance protocols with water, 70% ethanol, and/or 1% Tergazyme to remove clogs.
11. Some cell types may not stand the 1-h incubation in PBS. If cells become unhealthy, membrane integrity may be compromised, and calcein will be released into the solution and lost during the following wash step. Therefore, it is critical to test the loading time for specific cell lines and, if required, to adjust the calcein concentration and incubation duration to achieve optimal results. Alternatively, phenol red-free medium can be used instead of PBS, which in some cell types is better tolerated (T. Gilbert, personal communication).
12. The wash steps promote the reduction of background calcein fluorescence possibly generated by spontaneous hydrolysis of calcein-AM, hydrolysis by residual esterases from serum-supplemented culture medium, or esterases released by a small fraction of cells that are damaged during the calcein-AM loading.

13. The described order of addition for DHT and compounds ensures that the cells will be exposed to both simultaneously. However, investigators can also explore an alternative sequence of addition, i.e., pinning compounds into wells of seeded cells, followed by addition of DHT. This alternative sequence requires one additional day for cell seeding per operation cycle, and, without good scheduling software or planning, this alternative sequence may result in variable times for cell preincubation and compound treatment before ligand stimulation. However, the alternative sequence may help reduce (but not eliminate, due to the relatively long incubation time) the chances of interference with cell attachment by certain compounds. Investigators are advised to balance these pros and cons in selecting the optimal sequence of events based on the properties of cell lines and compound libraries to be used in their specific screens.
14. Sometimes the YFP moiety of a CFP-YFP fusion will generate signal in the FRET channel due to the overlap of the excitation spectra of CFP and YFP, and the physical bandwidth of the excitation/emission filters and the dichroic mirror. This phenomenon is referred to as bleed-through and is instrument-dependent. The amount of bleed-through needs to be subtracted from the total FRET signal. The degree of bleed-through can be determined experimentally for an instrument when a pure YFP sample is measured for its intensities via the instrument's YFP and FRET channels. The fraction of bleed-through is the ratio of signal from the FRET channel over that from the YFP channel.

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